Gene Expression Profiling during the Development of Testicular Hypertrophy in Neonatal Hypothyroid Rats

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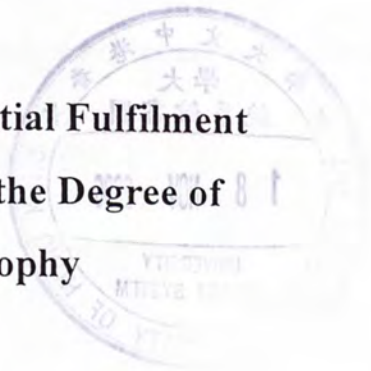

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Abstract of thesis entitled:

GENE EXPRESSION PROFILING DURING THE DEVELOPMENT OF TESTICULAR HYPERTROPHY IN NEONATAL HYPOTHYROID RATS

submitted by **TAO Kin-pong**, Department of Physiology,

for the degree of Master of Philosophy at The Chinese University of Hong Kong

Abstract

Spermatogenesis is a well coordinated process which involves complex interactions between Sertoli cells and developing spermatogonia. During the migration of germ cells from basal side to adluminal compartment of seminiferous tubule, extracellular matrix and intracellular cytoskeleton were under a continuous dynamics with constant assembly and disassembly of structural and adhesion molecules. While maintaining an adhesive force is essential to keep differentiating cells attaching onto the Sertoli cell lining, a degree of slack is also needed to allow pushing the matured sperm across the epithelium. These two counteracting actions must be adjusted in a fine manner, yet the molecular mechanism is still an unanswered paradox. Proteases, protease inhibitors and matrix molecules could be crucial factors controlling the normal development of mammalian testis.

Transient neonatal hypothyroidism is known to result in a paradoxical enlargement of adult testis by extending the proliferation periods of Sertoli cells. In this study, hypothyroidism was induced by giving 0.1% propylthiouracil (PTU) in

rats' drinking water between postnatal Day 4 to 24. Transcriptional profiles of proteases and protease inhibitors, especially the family of An Disintegrin and Metalloproteases (ADAMs) and Tissue Inhibitors of Metalloproteases (TIMPs), were examined extensively by using Real-Time RT-PCR. Cytokines, matrix adhesion proteins and small GTPases genes were also examined. Temporal gene expressions of PTU-treated litters were compared with that of control at postnatal day 24, 30, 40, 50, 60 and 90 aged testes. Result showed that hypothyroid rats suffered a significant down-regulation of most studied genes before day 40 or day 50, but all recovered back to normal level afterwards. Gene expressions under different treatment regimens by changing the concentration and duration of goitrogen administration have also examined.

Data obtained from this model of testicular hypertrophy share extreme coherence with the gene clustering data generated from a recent micro-array study on the developmental regulation of testicular genes. Genes that were classified as "MEIOTIC" cluster (ADAM2, 3A, 5, Sert-1, Pki β etc) all suffered severe down-regulation under hypothyroidism before day 40, while genes that extended this down-regulation up to postnatal day 50 were categorized into "POST-MEIOTIC" (ADAM 4, 6 ACE, Cystatin 8, etc). Interestingly, genes like Col3α1, Id-2, GATA-4 and many GTPases which were grouped as "MITOTIC" cluster do not show any unique temporal expression pattern. Lastly, transcription of "SOMATIC" genes are always not time or treatment dependent. Specific expression patterns drew from this clustering idea proved that particular types of genes within same cluster should have their own critical period of action. Profiling of transcriptome may help to predicate their role during the development of mammalian testis.

摘錄的題目名爲：

新生期間甲狀腺功能不足引至大白鼠睾丸肥大過程中之基因表達譜

摘錄

精子發生是一個具備良好協調的過程，它需要足細胞與生殖細胞之間的複雜交互作用。當生殖細胞從輸精管的底層移往內腔室的時候，細胞外的基質及細胞質骨架都處於持續的動態環境中，並不斷合成及分解結構性分子和黏附分子。分化中的生殖細胞黏附在足細胞的同時，亦要使已成熟的精子通過輸精管表層。雖然其分子上的調控機制還未十分清楚，這兩個相互頡頏的作用必須具備一個完善的機制去調節。蛋白酶，蛋白酶抑制劑以及其他基質分子均可能是哺乳類睾丸發展的重要因素。

短暫性的新生期甲狀腺機能不足症已知能透過延長足細胞增生期而引起成年期睾丸肥大。在這個研究中，母鼠出生後第四日到第二十四日之間在其飲用水中添加零點一個百分比的丙基硫尿嘧啶，以誘發甲狀腺功能不足症。利用實時逆轉錄聚合酶連鎖反應技術檢測蛋白酶以及蛋白酶抑制劑，特別是解整聯蛋白及金屬蛋白酶（ADAM）及金屬蛋白酶的組織抑制物（TIMP）的轉錄譜，並分析組織介素，細胞質黏性蛋白及小鳥苷三磷酸酶。經丙基硫尿嘧啶處理過的鼠組，與正常的鼠組在出生後第２４，３０，４０，５０，６０及９０日進行時間性的基因表達譜比較。結果發現甲狀腺功能不足的大鼠於第四十至五十日間基因表達大幅下調，但於第六十日之後回復正常水平。本實驗還研究了甲狀腺素歸還治療，及更改致甲狀腺腫物的濃度和給藥時間等不同情況下的基因表達。

是次利用睾丸肥大作模型的實驗結果與一個近來以微矩陣得出的群叢研究相當吻合。被歸類爲「減數分裂性」的基因（ADAM2, 3A, 5, Sert-1, Pki β 等）都因甲狀腺功能不足而於第四十日前受到較大的減控，而所有被歸類爲「後減數分裂性」的基因（ADAM 4, 6, 血管緊張肽轉變酶, 半胱氨酸蛋白酶抑制劑八等）更甚至延長其減控至第五十天。值得注意的是某部分被歸類爲「有絲分裂性」的基因如 Col3α1, Id-2, GATA-4 及很多鳥苷三磷酸酶，它們基因表達並沒有特定的時間性規律。「體細胞性」的基因通常也都沒有時間或經實驗處理的依賴性。從是次群叢研究的結果可發現特定的基因群叢具有其指定的臨界作用週期。基因轉錄的圖譜研究可幫助了解它們在哺乳類睾丸發育過程中的作用。

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Abbreviations

α_2MG	Alpha-2 Macroglobulin
3β-HSD	3 beta-hydroxysteroid dehydrogenase
ABP	Androgen Binding Protein
ACE	Angiotensin Converting Enzyme
Act	Activator of CREM in testis
ADAM	A Disintegrin and Metalloprotease
ADAMTS	A Disintegrin and Metalloprotease with thrombospondin repeats
ANOVA	Analysis of Variance
β_2MG	Beta-2 Microglobulin
BTB	Blood-Testis-Barrier
cAMP	Adenosine-3', 5'-cyclic monophosphate
cdc42	cell division cycle 42
Cdh	Cadherin
CDKI	Cyclin-dependent kinase inhibitor
cGMP	Guanosine-3',5'-cyclic monophosphate
Cip1	Cyclin-dependent kinase inhibitor with Mr of 27kDa
Clgn	Calmegin
Cx43	Connexin 43
Col3α1	Collagen type III alpha 1 subunit
CREB	Cyclic AMP response element binding protein
CREM	Cyclic AMP response element modulator
Dncic1	Dynein, cytoplasmic, intermediate chain 1
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGF	Epidermal Growth Factor
Eppin	Serine protease inhibitor-like, with Kunitz and WAP domain 1
ER	Endoplasmic reticulum
FSH	Follicle Stimulating Hormone
GATA-4	Zinc finger proteins that bind to A/T GATA A/G consensus sequence -4
GnRH	Gonadotrophin Releasing Hormone
GTPase	Guanosine Triphosphatase
Id	Inhibition of differentiation proteins
Kip1	Cyclic-dependent kinase inhibitor with Mr of 21kDa
KRP	Kinesin-related Protein
LDHc	Lactate Dehydrogenase 3, C chain

LH	Luteinizing Hormone
MGP	Matrix Gla Protein
MMI	Methimazole
MMPs	Matrix Metalloproteases
MT-MMP	Membrane Type - Metalloprotease
Nedd4a	Neural precursor cell expressed, developmentally down-regulated gene 4a
Nep	Neprilysin
NT3	Neurotrophin 3
Odf	Outer dense fibre protein
PAs	Plasminogen Activators
PACAP38	Pituitary Adenylate Cyclase Activating Polypeptide 1 (Adcyap1)
PACAPr	Adenylate cyclase activating polypeptide 1 receptor 1 (Adcyap1r1)
PAI	Plasminogen Activators Inhibitors
PCI	Protein C Inhibitor
Pcsk4	Proprotein convertase subtilisin/kexin type 4
Pki β	Protein Kinase Inhibitor beta
Prm	Protamine
PRSS21	Protease, serine 21/ testisin
PTU	Propylthiouracil
Ras GRP-1	Ras guanyl releasing protein 1
Rhes	Ras homolog enriched in striatum
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription - Polymerase Chain Reaction
Sert-1	Sertoli cell protein 1
Sp10	Sperm Protein 10, Acrosomal vesicle protein 1
Spam	Sperm adhesion molecule
Spink2	Serine protease inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)
Sycp	Synaptonemal complex protein
T_4	Thyroxin
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloprotease
tMDC	tissue-type, metalloproteinase-like, disintegrin-like, cysteine-rich protein
TNF	Tumor Nerosis Factor
TPs	Transition Proteins
TRs	Thyroid Hormone receptors
$UV_{260/280}$	Ultra Violet at the wavelengths of 260 and 280 nm
Zfp37	Zinc finger protein 37
ZO	Zonula Occludens protein

Introduction

Mammalian testes are responsible for the production of male gamete spermatozoa, and secretion of male sex hormone testosterone, which stimulate the development of masculine sexual characteristics. Whole testis is enclosed within a capsule *tunica albuginea* and muscle layers. The major constitutes of testes are the labyrinthine system of seminiferous tubules, and the surrounding interstitial tissues (interstitium).

Interstitial tissue and Leydig cells

The interstitial compartment contains blood and lymphatic vessels for nourishment of testicular cells. Apart from the connective tissue and lymphatic macrophages, the most important constitution of interstitial compartment is the Leydig cell. Leydig cells are responsible for the secretion of androgens that stimulate the differentiation of male tract during fetal life. During the development from fetus to adult, Leydig cells undergo transformation and proliferation until Day 60[1], when the number of Leydig cells remains constant and synthesis testosterone as their major secretion.

Seminiferous tubules

The convoluted seminiferous tubules make up more than 70% of adult rat testis. These tubules are covered by thick basal lamina and layers of peritubular myoid cells. The contractile myoid cells provide mechanical tension to expel the sperm together with fluid through the lumen[2]. The basement membrane is a specialized organization

of connective tissue that assists in providing solid support for the tubule. The lumen of seminiferous tubules is lined with the seminiferous epithelium which contains two general types of cells: spermatogenic germ cells and Sertoli cells.

Male germ cell line and spermatogenesis

Spermatogenesis is the process of male gamete production in which spermatogonia developed into spermatozoa. This progress can be divided into three developmental phases with characteristic stages of germ cells: Mitotic phase (spermatogonia), Meiotic phase (spermatocytes) and Post-meiotic phase (spermatids)[3].

1. Mitotic phase (Proliferative phase)

Mammals produce millions of sperms per day. In order to replenish the germinal epithelium to sustain a constant number of available cells, a self-renewal mechanism is required. The first member of spermatogenesis is the *stem spermatogonium*, which is located nearest to the wall of seminiferous epithelium attaching onto basement membrane. In rats these cells appears as early as postpartum Day 4[4] and can be further divided into two types: Type A spermatogonia are isolated and proliferative stem cells that are responsible for new generation of both Type A and B spermatogonia, while Type B spermatogonia are actively dividing and differentiating that are responsible for the formation of spermatocytes. At postpartum Day 6[4], rat spermatogonium starts to differentiate and undergo a series of mitosis during which the nuclear compartment divided but remains cytoplasmic

continuity known as intercellular bridges[5]. At the end of mitotic phase, matured portions of spermatogonia (type B) are developed to form young diploid primary spermatocytes.

2. Meiotic phase

After the post-mitotic differentiation, these primary spermatocytes enter the S-phase of cell cycle, arrested and become *Preleptotene Spermatocytes*. This meiotic phase is an extended and long-lasting period. Within this three-week period of time, the spermatocytes undergo gradual morphological transformations with sizes of cytoplasmic content increases progressively that can be easily identified under light microscopy. According to their nuclear histological organizations these spermatocytes can be further developed into *leptotene, zygotene, pachytene and diplotene spermatocytes* ,with their first time of appearance ranging from Day 9, 15, 18 and 26 postpartum respectively[4]. Leptotene cells start the prophase of meiotic by condensation of chromosomes. During the transition of preleptotene to leptotene spermatocytes, these cells migrate from the *Basal* to the *Aluminal compartmen*t of testis, where the intercalated *Intermediate compartment*[6] is the site where tight junction of Sertoli cells located. Homologous chromosomes are paired in zygotene cells with the help of synaptonemal complex[7], and the event of genetic recombination, crossing over, is initiated within pachytene cells. Together with the last diplotene cells, these mentioned diploid primary spermatocytes finish the prophase and rapidly complete the remaining metaphase, anaphase and telophase to ends the Meiotic I. After the first meiotic division, which is also known as

reductional division, two homologous chromosomes within each primary spermatocyte are separated and become two haploid secondary spermatocytes. Cells eventually enter the second meiotic division, Meiosis II or equational division, to produce four identical haploid *Round Spermatids* when the prolonged meiotic phase ends.

3. Post-meiotic phase (Spermiogenic Phase)

This terminal process of spermatogenesis is also celled spermiogeneis. During this process, the round spermatids undergo massive cytoplasmic transformation to develop into completely matured spermatozoa. Cell volume is significantly reduced with the development of flagellum and acrosome. Characteristic shape of sperm is formed by nuclear condensation and elimination of cytoplasm. Spermatozoa are ready to be released upon activation for fertilization of ovum.

During the progress of spermatogenesis, the developing germ cells always remain attached and being surrounded by irregular-shaped Sertoli Cells.

Sertoli Cells

The Sertoli cells constitute up to 15% of testis content and contribute a very important role in assisting the establishment of spermatogenesis. The main function of Sertoli cells is to provide nutritive and mechanical support to hold the developing germ cells within its luminal crypts. It also secretes inhibin and activin, which regulates the positive and negative feedback on Follicle Stimulating Hormone (FSH)

level. Another important function of these cells is the formation of blood-testis barrier (BTB). Within the testis, there are specialized types of cell junctions that are unique around the whole body. Two adjacent Sertoli cells are linked together by tight junction. This barrier can restrict the entries of limited substance but still allow the diffusion of nutrients towards germ cells. Though having a great surface area, Sertoli cells have limited capacity on supporting the development of sperm, meaning that each cell can only sustain a particular number of germ cells to growth[8]. In case of rats, the Sertoli cell to germ cell ratio is around 1:50. This suggested that the potential adult sperm production is heavily influenced by the number of Sertoli cells present on developed testis. While the importance of these cells is highly concerned, its proliferative period is relatively short. Sertoli cells are terminally differentiated cells that their amount will not be replenished upon lose. In rats, Sertoli cells cease dividing as soon as at postpartum Day 15[9], thus increasing the mitotic activity during this period or extension of proliferation period is a remedy for increasing the fertility of adults in terms of sperm count.

Specialized organizations of junction present in testis

As mentioned before, testis has unique junctional arrangements that are distinctive from other organs for communicating between cells and their surroundings. It is now demonstrated that three morphologically and functionally apparent types of cell junctions are present in the seminiferous epithelium[10]. The first one is occluding junction[11], which are generally called tight junction. Tight junction is formed between two adjacent Sertoli cells which divides the seminiferous epithelium into two permanents (basal and adluminal) and one transient (intermediate) compartment. The basal compartment contains spermatogonia and early stage of leptotene spermatocytes.

Within this compartment, cells bear free access of substances from blood and the lymphatic circulation. Beyond the basal compartment is the adluminal compartment where the spermatocytes and spermatids reside. After meiosis, spermatids are haploid which are immunologically different with that of diploid cells. Thus tight junction is essential in blocking the circulating antibodies or foreign substances that may damage the developing germ cells. There is another putative intercalated partition named as intermediate compartment which will be discussed below. The second type of cell junctions is the anchoring (or adhering) junction, which can be further classified into four main groups depending upon their nature and localization. These actins based or intermediate filament based junctions are present between Sertoli cells, germ cells and the extracellular matrix. Their proposed function is to link the cells' internal machinery or cytoskeleton to another cell or matrix in establishing a trafficking network and maintain tissue integrity. The third type of cell junctions is the gap junctions[12], which are intercellular channels that allow exchange of ions and small molecules between two cells. There are also testis specific junctions like ectoplasmic specialization[13] and tubulobular complexes[14] that hold the spermatids to avoid premature release.

Dynamic nature of Sertoli-Sertoli & Sertoli-germ cell junctions

Because of the high variety of junctions present in testis, a controlled mechanism is required to protect these machineries to function, especially during spermatogenesis. In order to escape from the immunogenic attack, cells need to migrate from basal compartment towards the adluminal compartment. It is supposed that within the transient intermediate compartment, leptotene spermatocytes or Sertoli cells need to secrete proteins that assist in successive formation and breakdown of tight junction to

retain the integrity of blood-testis barrier. During the germ cell development, there is a unipolar migration of spermatocytes that needed to provide space for the continuous proliferation from basal spermatogonia. The mechanism of how the germ cells transversing across the densely packed epithelium is still an unanswered paradox. To achieve this migration, extracellular matrix and intercellular cytoskeleton were under a vigorous dynamics. Non-static disassembly and assembly of structural and adhesive molecules is required to remodel the microenvironment for conveying cells. During the migration, an adhesive force must be maintained to hold the differentiating spermatocytes attaching onto Sertoli cell lining for nourishment and protection, but at the same time a degree of stringent is also essential to allow pushing the matured spermatids away from basal compartment. These two counteracting forces must be fine-tuned in an organized manner: If the adhesion is too strong, developed spermatozoa cannot be released into lumen and will be phagocytized by Sertoli cells eventually. In contrast, undeveloped spermatocytes will be discharged prematurely that lost nutritive support and failed to fertilize an ovum.

Role of proteases and protease inhibitors in male gametogenesis

In order to adjust the microenvironment during spermatogenesis, either the secretory Sertoli cells or the developing germ cells need to release proteins that support matrix remodelling. Noticed that genes required for spermatogenesis and fertility often encode transcripts present in meiotic or post-meiotic germ cells but not in somatic tissue[15]. Proteases are well-known for their involvements in cell movement of metastasis and invasion in cancer cells[16]. Some members of protease are also important in the pathophysiology of extracellular matrix related disorder like arthritis, yet the role of protease in testicular development is still unclear. Proteases can be

classified into four main classes according to their catalytic types: Cysteine, Aspartic, Serine and Metallo-peptidases. Both Sertoli cells and spermatogenic cells express intensive abundance of protease and protease inhibitors, but most of their specific substrates are still unknown. The high versatility of proteases may help remodelling by digestion of matrix proteins, or activating cytokines and receptors for signal transduction. To avoid non-specific degradation, proteolysis must be controlled at specific site, thus specific protease inhibitors are required. It is important to evidence here that during tissue remodelling, proteases are only activated at specific site of action, while the activity at other sites is always suppressed with presence of inhibitors. Some members of proteases [like tissue Plasminogen Activators (tPA) and Cathepsins] and protease inhibitors are well characterized for their contributions in spermatogenesis, but another family of growing awareness are the A Disintegrin and Metalloproteases (ADAMs).

Proteases and Proteases Inhibitors expressed in testis

ADAM is a growing member of metalloprotease which belongs to the zinc protease family, metzincin subgroup. There are seven dedicated domains for different putative functions: (1) Pro-domain, which activates the ADAMs upon cleavage. (2) Metalloprotease domain, which perform shedding and cleavage of proteins. (3) Disintegrin domain, which bind with integrin subunits. (4) Cysteine-rich domain, which supports syndecan-mediated adhesion. (5) EGF-like domain, which stimulates membrane fusion in some ADAM candidate. (6) Trans-membrane domain, where the ADAM protein anchors on (7) Cytoplasmic tail, which binds with different signalling molecules and receptors[17]. Up-to-date, there are at least 39 ADAMs genes identified in different species, among which at least 15 of them are testis specific or

predominantly expressed. The intensified abundance expressed in a sole organ proven that the role of ADAMs in testicular development should not be underestimated. Another closely related family is the A Disintegrin And Metalloprotease with Thrombospondin-like motifs (ADAMTS). ADAMTS still possess Pro-domain, metalloprotease and disintegrin domain but lack the remaining domains, replace by repeats of thrombospondin-like sequence. These repeats may mimic the function of adhesive domains that ADAM bears, since thrombospondin is know to interact with cell surface molecule- sulfated glycosaminoglycan. The functions of some members of ADAMTS have been described for long. For example ADAMTS-2, which is previously called Procollagen N-proteinase, is responsible for proteolysis of type I and II collagens – the main component of extracellular matrix. Under the metzincin subgroup (where ADAM and ADAMTS belongs to), there are also matrix metalloproteases (MMPs)[18] which are enzymes that responsible for degradation of matrix and wound healing. Testis also expresses high level of protease inhibitors, for example the Tissue Inhibitors of Metalloproteases (TIMPs), which known to suppress the activity against a wide range of metalloprotease, and cystatins, specific inhibitors of cysteine proteases like the cathepsins. Common protease inhibitor like α2-macroglobulin (α2MG), together with other specific inhibitors may help protecting against the attack of proteolysis done by ADAMs.

Hormonal control of spermatogenesis

Hormonal control over the male gamete development has been studied extensively over the past half century, especially the example of FSH. From different experimental designs (hemicastration, GnRH antagonist or recombinant FSH treatment)[19], it is well known that FSH can directly enhance fertility through its

mitogenic activity[20]. During the fetal development of rat, FSH stimulates the division of both Sertoli cell and spermatogonia during the proliferative phase, thus increasing the testicular mass and number of Sertoli cells at the adult stages. Sertoli cell will also produce inhibin and activins that feedback to the pituitary to inhibit the secretion of FSH. Another known pituitary hormone that helps sperm production is the Luteinizing Hormone (LH). LH stimulates the production of testosterone from Leydig cells, which is an essential component in generating a microenvironment of Sertoli cells for gamete differentiation. Yet the physiological control of FSH, LH and testosterone is well characterized[21], the role of thyroid hormones on testis development is not clearly understood because of the paradoxical distributions of thyroid hormone receptors (TRs) among each testicular cell types. Classical studies suggested that adult testis is unresponsive to thyroid hormone due to the undetectable expression of any TRs on the entire adult testis[22]. Recent RT-PCR and immunochemistry studies revealed that members of TRs expressed in particular period during the development[23], while Sertoli cell is regarded as the main target of thyroid hormone in testis. Thyroid hormone receptor (TRα1 in particular) for example only expressed in early stages of developing spermatocytes (from intermediate spermatogonia to pachytene spermatocytes). More interesting findings uncovered that expression of TRα1 on Sertoli cells is only limited at the time when they are dividing, and their expressions started to be eliminated after the cessation of proliferation period of Sertoli cells. This clearly states that thyroid hormones play an important role on the proliferation of Sertoli and germ cells within a critical period.

Hypothyroidism and testis development

The use of neonatal hypothyroidism as a model of studying testis growth is well established and its effect is considerably great. Induction of neonatal hypothyroidism by 0.1% propylthiouracil (PTU) can increase the number of Sertoli cells and testis mass in adult by 182-257% and 127-162% respectively[24]. Other parameters reflecting the fertility also increased significantly (for example the interstitial volume increased by 60%, while Daily Sperm production has elevated by 140%[25]). In contrast, hyperthyroidism causes a lost of 50% of Sertoli cells with half-weighted testis[26]. Propylthiouracil is a reversible goitrogen. Upon ingestion by mother rats, it passes through milk and causes severe hypothyroidism in pups.[27] However the use of PTU as low as 0.006% could increase testis size and sperm production maximally with minimized side effects.[28] Hormone profiles from radioimmunoassay studies shows that under 0.1% PTU treatment, testosterone level is normal[29], while FSH and LH were 40%-50% lowered despite the increases in testis size and sperm count.[30] Together with the TRs expression profiles, these data indicate that thyroid hormone may play an indirect role in inducing the increase in Sertoli cell number, but not in a mitogenic manner. Thus it was proposed that hypothyroidism may slow down the maturation of Sertoli cells, which extends the available time for mitosis[31]. This means that thyroid hormones may indirectly stimulate the differentiation of Sertoli cell in neonatal period, with their actions independent from FSH, LH or testosterone. It was even proposed that the disappearance of mild perinatal hypothyroidism (due to the adequate intake of iodine) may be involved in the reduced fertility in urbanized citizens when compared with that of the starved.

Genes to be studied:

In order to identify genes that may be affected by hypothyroidism during rats' postnatal development, a temporal expression profiling of different genes were carried out. Proteases and their endogenous inhibitor are especially emphasized.

Proteases:

ADAM 2, which is previously named as fertilin β or PH-30, is well known for its role in sperm-egg fusion during fertilization. Upon dimerization with ADAM 1, the fertilin complex on sperm is able to interact with α6β1 integrin on egg surface for membrane fusion[32]. Knockout studies have shown that rats lacking ADAM 2 expression are infertile but healthy, while the sperm suffered an eight fold decrease in binding capacity to egg[33].

ADAM3, which is also named as cyritestin or tMDC I (tissue-type, metalloproteinase-like, disintegrin-like, cysteine-rich protein I), is first characterized as a trans-membrane protein involved in fertilization. Cyritestin was another testis-specific ADAMs that proposed to be a sperm protein involved in gamete interaction and sperm-egg plasma membrane adhesion and fusion[34].

ADAM4 (tMDC V) is predominantly expressed in testis, but low abundance of transcription is also found in other tissues like heart[35]. Inhibitory loop peptide analogy with ADAM 4 has no effect in affecting the sperm-egg fusion[36], which means this gene may participate in dual proteolysis and integrin-mediated cell-cell, cell-matrix interaction.

- **ADAM5** (tMDC II) is first characterized as a testis-specific gene that was identified together with ADAM 4 and 6 from a screening of mouse cDNA library in testis. With increased sensitivity, low transcription was also detected in brain, kidney, and ovary. **ADAM 6** (tMDC IV) is predominantly expressed in testis, and detectable expression is also present in prostate.

ADAM15, also called Metargidin or MDC 15, was isolated from a human mammary epithelial carcinoma cell line and is widely expressed[37]. About half of the presently known metalloprotease disintegrins carry a catalytic site consensus sequence for metalloproteases (HEXXH) and are therefore predicted to be catalytically active, whereas the remaining family members do not contain a catalytic site in an otherwise related metalloprotease-like domain and therefore must lack metalloprotease activity. ADAM 15 is one of the members of ADAMs that bear active metalloprotease activity[38], and is known for its ability interacts with αvβ3 integrin.

ADAM18 (tMDC III) is a protein processed on the sperm surface during epididymal transit[39]. From human orthologues studies revealed that ADAM 18 contains putative integrin-binding Glu-Cys-Asp (ECD) motif[40] that may facilitate oocyte recognition during fertilization similar to ADAM 2 and 3.

ADAM30 was identified by Northern Blot against human testis cDNA library, where its expression is testis specific[41]. The identification of this novel gene is new and thus its role in fertilization or spermatogenesis is not yet characterized.

ADAM32 is one of the most-recently identified members of ADAMs family. It is known that ADAM 32 gene is predominantly expressed in the testis. Northern Blot

and RT-PCR data revealed that expression of ADAM 32 begins during the meiotic stage, where pachytene spermatocytes are their major expression location[42].

ADAMTS-1 (METH1) is well known for its inhibitory effect on angiogenesis due to its thrombospondin-1 repeats. It is an active protease associated with ECM and essential for normal growth, fertility, and organ morphology. Unlike the transmembrane ADAMs family that cleaves membrane surface proteins, ADAMTSs are soluble, secretory extracellular matrix proteins, thus substrates that can be processed by ADAMTSs are rather well-illustrated. There are matrix metalloprotease (MMP) cleavage sites in its spacer domain[43], thus the interaction with other proteases upon activation is proposed. With its high homology with ADAMTS 4, ADAMTS 1 is able to cleave cartilage proteoglycan, aggrecan and versican at similar sites.

ADAMTS-4 is also called aggrecanase-1. ADAMTS-4, together with ADAMTS-5/11, is regarded as aggrecanase that involved in degradation of aggrecan, a cartilage protein involved in the pathophysiology of arthritic diseases. Its activity of proteolysis can be regulated by other proteinases or inhibitors. For example, it can be activated by another family of proteases, the MT4-MMP[44]. But can also cleave the global protease inhibitor α_2MG[45], or inhibited by TIMPs, especially TIMP-3.

ADAMTS-5 (aggrecanase-2) cleaves aggrecan at different sites[46]. This ADAMTS-5, together with TS-1 and TS-4, has been shown to degrade members of the lectican family of proteolycan[47], suggesting that they have important roles in the remodelling of ECM. Notice that thyroid hormones can up-regulate the expression of ADAMTS-5[48], suggests that thyroid hormones may assist in degradation of ECM components indirectly under the regulation of secretory proteases.

ADAMTS-16 was first cloned in human cDNA library, where lung, kidney, brain and ovary are the main encoding location[49]. Here we report the temporal transcription of putative ADAMTS-16, with the primer sequence based on the mice homolog.

The matrix metalloproteinases (MMPs) are zinc metalloproteases that can possibly degrade all structural components of the extracellular matrix like type III collagen, proteoglycan, gelatine and fibronectin. This family of endopeptidase is also able to active a number of cytokines and other proteases, thereby their role in developmental physiology in tissue remodelling is well known. MMPs can be divided into secreted from or membrane-type form. The proteolytic activity in latter form (MT-MMP) can be regulated by TIMPs and is highly specific. **Membrane type 3 - Matrix Metalloprotease (MT3-MMP),** which is also called MMP-16, is shown to activate another potential protease, pro-MMP2. This cleavage is subjected to inhibition to TIMP-2 and -3, but not -1[50]. It was shown that upon autocatalytic processing and ectodomain shedding, MT3-MMP is potent to digest type III collagen than other MT-MMP members.

The metalloprotease **Angiotensin I Converting Enzyme (ACE)** is an ectoenzyme anchored on plasma membrane that plays a pivotal role in the cardiovascular system by generating the vasoconstrictor peptide angiotensin II[51]. In the mammalian testis, two isoforms, namely somatic and germinal ACE are known. Although both forms are present in testis, the testicular isoform (tACE) is exclusively expressed during spermatogenesis. A soluble form of ACE can be derived from membrane-anchored form, which can be found in many biological fluid like seminal fluid and amniotic fluid. It was shown that mice lacking ACE expression suffered a reduced fertility by failure in oocyte recognition. But its best-known substrate,

angiotensinogen, is not fertility-related[52], indicating that other substrates maybe involved in the role of spermatogenesis by ACE.

Neprilysin (Nep) is a transmembrane ectopeptidase under the zinc metalloprotease family, where its main expression is localized on the brush-border membrane of kidney. Nep bears conserved cysteine residues in extracellular domains, which may be essential for the maintenance of its structure and function upon activation by other proteases[53]. Its substrate includes vasodiliators such as atrial natriuretic peptide, and some neuropeptides like substance P. No natural inhibitors of Nep have been identified, in which TIMPs cannot exert its inhibitory effect on it. Its biological importance in reproduction is not yet characterized, but Nep is down-regulated in prostate cancer to facilitate the tumour progression, possibly by the stimulation of mitogenic peptides[54].

PRSS21/Testisin is a recently characterized homolog of human serine protease. PRSS21 was abundantly expressed in testis and lost in testicular tumors, thus it was considered as a putataive tumour suppressor gene. Immunostaining revealed the expression is limited to the cytoplasm and plasma membrane of premeiotic germ cells[55]. The physiological peptide substrates have not been identified, but mice PRSS21 shows gelatine-hydrolysing ability that proposed to assist fertilization[56].

Plasminogen Activators (PAs) are specific serine proteases that catalyze the conversion of plasminogen into plasmin, a protease with trypsin-like substrate specificity. Some specific factors of the PA system are known: the activators, **tissue type activator** (tPA) and **urokinase type activator** (uPA) whose activities are controlled by two **serine plasminogen activator inhibitors**, PAI-1 and PAI-2. Sertoli

cell cultures secrete plasminogen-dependent proteases into medium that can be regulated by FSH level, thus PAs are extensively studied for their roles in fertility by Sertoli cells. Major constitutions of PAs are synthesized by Sertoli cells, but still a lower amount of expression can be detected in spermatocyte extracts.

Proteases Inhibitors:

Activities of the ADAMs, ADAMTSs and MMPs can be inhibited principally by the four **Tissue Inhibitors of MetalloProteinase (TIMPs)**. For example, TIMPs inhibits most of the MMPs in a 1:1 stoichiometric ratio[57]. Each member of TIMPs has high specificity and independent inhibitory activity over different metalloproteases. In case of **TIMP-1** as an example, it potentially suppresses activity of all MMPs, but a reduced capacity on inhibiting some matrix-bounded family members is shown (MMP-14, -15, -16, -24)[58]. One of the most well-known inhibitory targets is Gelatinase B (MMP-9), which suggested that TIMP-1 play important role in processes that involves tissue remodellling like tumour invasion and organ morphogenesis.

Apart from the inhibitory effect against peptidase, it was proposed that **TIMP-2** can also act as a receptor. The binding of TIMP-2 to MT1-MMP can form a complex that acts as a receptor for pro-Gelatinase A (MMP-2) for activation to digest a great variety of collagens. Expression of these molecules is modulated by FSH and is therefore its role in spermatogenesis is implicated[59].

Unlike TIMP-1 and TIMP-2 which is in a soluble form, **TIMP-3** is an ECM-associated protein with distinct expression with that of the previous ones[60]. Similar with other members, TIMP-3 is also able to inhibit all known MMP family

members[61]. Studies pointed out that TIMP-3 maybe involved in the morphogenesis of certain epithelial structures, and in placental implantation[62].

TIMP-4 was first cloned in human cDNA library and is abundantly expressed in heart[63]. Its transcription in other parts is restricted, where very low level was detected in the kidney, placenta, colon, and testes, suggested that TIMP-4 may play tissue-specific inhibition with is distinct with other TIMPs. Unlike TIMP-3 which has a unique association with the ECM, TIMP-4 binds to the COOH-terminal hemopexin-like domain of MMP-9[64], and both TIMP-4 and MMP-9 were expressed in both Sertoli cells and gonocytes[65].

Alpha-2 macroglobulin (α2MG) is a secretory product of Sertoli but not germ cells. Its uniqueness is reflected by its ability to inhibit the proteolytic activity of all classes of proteases and is widely expressed. In testis, it was localized to Sertoli cell cytoplasm and around the heads of elongating and elongate spermatids possibly at the site of apical ectoplasmic specialization, suggestive of its significance in germ cell movement and spermiation. Neither FSH nor androgens affect the secretion, thus it was postulated that α2MG might limit unwanted proteolysis to maintain the integrity of Sertoli cell barrier, since extensive cytoplasmic remodellling in the seminiferous epithelium is continuous during spermatogenesis[66].

Bikunin, also called urinary trypsin inhibitor (ulinastatin) is a Kunitz-type serine protease inhibitor that is first characterized in cancer cells. Together with α1 microglobulin; bikunin is produced by post-translational processing of their common α_1-microglobulin/bikunin precursor[67]. Bikunin has two Kunitz-type proteinase inhibitor domains, and is proposed as a main participant in suppression of tumor cell

invasion and metastasis possibly through inhibition of urokinase-type plasminogen activator (uPA) and limit collagen synthesis.

Cystatins are specific, potent and reversible inhibitors of the C1 family of papain-like cysteine proteases, including the mammalian cathepsins B, H, L, and S[68]. **Cystatin 8**, which is also named as cystatin-related epididymal spermatogenic protein, is predominantly expressed in proximal caput region of the epididymis, 20-fold more than in testis[69].

Cystatin C (Cystatin 3) is thought to be a member in the pathogenesis of Alzheimer disease by its amyloidogenic ability that colocalizes with beta-amyloid. Cystatin C displays the strongest inhibitory activity of all cystatins toward lysosomal cysteine proteases in general and has a widespread distribution in human tissues and body fluids, including cerebrospinal fluid, seminal plasma, and milk[70].

Cystatin-related gene expressed in Sertoli cells (Cystatin SC) and **cystatin-related gene highly expressed in testis and epididymis** (Cystatin TE-1) are recently identified members of cystatins superfamily by the use of differential display RT-PCR[71]. Cystatin SC was only detected in testis and Sertoli cell cultures by Northern blot analysis, while no apparent expression was found in spermatogenic cells or epididymis by *in situ* hybridization.

Unlike Cystatin SC, **Cystsatin TE-1** has a wider expression location. Northern Blot revealed that gene was also present in proximal epithelium of epididymis, and Sertoli cells. Weak signals were also detected in ovary and prostate but not in other tissues. But the expression of Cystatin TE-1 is absent in spermatogenic germ cells and

Leydig cells.

Protein C inhibitor (PCI) is a nonspecific, heparin-binding serpin (serine protease inhibitor) that inactivates many plasmatic and extravascular serine proteases by forming stable 1:1 complexes. As it's another name imply, Plasminogen inhibitor type-3 (PAI-3) are predicted to be involved in the restructuring events that characterize the testis throughout development. Proteases inhibited by PCI include the anticoagulant activated protein C, the uPA, and the sperm protease acrosin[72]. The main source of PCI is Leydig cells. Null mice lacking PCI expression are healthy but sterile in male with malformed sperm.

Other spermatogenesis related genes:

Calmegin (Clgn) is expressed in meiotic-germ cell, localized on ER membrane and is testis specific. In spermatogenesis, the Clgn protein is thought to act as a chaperone for proteins in transition from endoplasmic reticulum to the spermatid surface[73]. The chaperone function of clgn, with expression initiated in late pachytene spermatocytes, is though to assist in the post-translational folding of fertilin complex that assist sperm-egg interaction during fertilization.

Heparan sulfate proteoglycans are important candidate in regulation of cell-cell adhesion and signalling. Syndecans is one of them that most extensively studied in testis for their ability to bind with varies ECM molecules and messagers. Of which, **syndecan-4** is localized on focal adhesions and their association with actin cytoskeleton suggested that it may participate in remodelling of testicular environment[74]. It was suggested to be a co-receptor together with the integrins to

bring upon the cytoplasmic signalling and adhesive functions through PKC pathway[75].

Tight junction is a multi-molecular membrane specialization. Occludin, **claudins** and several associated peripheral proteins such as ZO-1, -2 and -3 are main integration components that forming the trans-membrane domains in Sertoli cell barrier[76]. Tight junction dynamics are supposed to be regulated by several signalling pathway like TGFβ and protein kinases A and C[77].

The cadherin superfamily represents a group of calcium-dependent cell-surface glycoproteins meditating cell-cell recognition and morphogenesis[78]. Cadherins are well known for its participation in adhering junctions which found between Sertoli cells or Sertoli-Germ cells[79]. The machinery of cadherins is dependent on two factors: calcium and binding with catenin. It was shown that in the lack of calcium, cadherins are inactive and susceptible to proteolysis[80]. Upon binding with β- or γ-catenins, the cadherin-catenin complex is able to anchor onto the cytoskeleton by α-catenin to facilitate the construction of adhering junction[81]. Here we characterize the putative homolog of mice **Cadherin-8 (Cdh8)** in rat testis, in which the encoding of it is strictly limited in developing CNS for mouse[82].

Synaptonemal complex protein 1 (Sycp1) encodes the major component of synaptonemal complexes. Synaptonemal complexes are structures that are formed between homologous chromosomes during meiotic prophase, which is a key candidate involving the chromosome pairing and genetic recombination.

- Physiological effects of thyroid hormones are mediated by thyroid hormone receptors (TRs) which also serve as nuclear ligand-modulated transcription factors upon activation. TRs are encoded by two genes, alpha and beta, which are subjected to alternative splice to form different isoforms. In testis, TRα1 is exclusively expressed in Sertoli cells and trace transcription is also found in germ cells, but its expression is largely limited in cells during developing state. Expression of TR beta isoforms is not consistent among different studies and is rather paradoxical. Together with other binding assay, **Thyroid Hormone Receptor α1 (TRα1)** is though to be the main target of thyroid hormone in testicular environment[83].

Objectives:

Through the study on temporal expression on a variety of protease and protease inhibitors, we would like to use the neonatal hypothyroidism as a model of study to investigate the developmental profile of these genes. This may help us to understand the role of these proteolysis-related genes in matrix remodelling and testicular development.

Materials and Methods:

Animal treatments and tissue collection:

Pregnant Sprague-Dawley rats were housed individually under controlled temperature (20±1°C) and photoperiod (12L/12D) with free access to rat chow and water. The animal care and experimentation procedures were done in accordance with Guide lines approved by the local animal experimentation ethic committee. Some litters were made hypothyroid by adding either 0.1% propylthiouracil [PTU; Sigma, St. Louis, MO] (w/v) in the mother's drinking water from day 4 to day 24. At day 24, at which time control pups were weaned, no further treatments were given. Rats from both control and PTU-treated litters used for studying the transcription of ADAMs and ADAMTSs genes were killed by rapid decapitation at days 24, 30, 40, 50, 60 and 90. Body and testis weights were measured. Testes were snap-frozen in liquid nitrogen and stored at -70°C until extraction of total RNA using the Qiagen RNeasy mini-kit (Hilden, Germany).

In order to investigate the expression of non-spermatogenic genes at neonatal period, control and PTU-treated rats that caged in same conditions were scarified at the age of day 8, 16 and 24 and subjected to other RT-PCR procedures as described before.

To validate our experiment if alternation of gene expression is thyroid hormone responsive, another set of experiment was carried out with a thyroxin (T_4) replacement group. Together with the control and hypothyroid groups as previously described, same litter size of PTU-treated rats were injected with one dose of 240 ng

T_4 per kilogram body weight subcutaneously at day 35. All three groups of rats were killed at day 40 or day 50 and subjected to further procedures as mentioned.

Using the same laboratory setup, other six groups of pups were caged and cared under same conditions. Different induction of hypothyroidism was applied by changing the concentration, exposure duration and goitrogen that applied to mother rats' drinking water. In the first two groups, mothers were fed with tap water and 0.1% PTU from postnatal day 4 to 24 respectively as previously mentioned for comparison. In the third group, same concentration of PTU was applied with shortened duration from postnatal day 4 to 16 only to study its period of action. In the remaining groups, another goitrogen, methiamzole (MMI) was used. In the 4th group, 0.05% MMI was administered from Day 4 to 24 postnatally in mothers' drinking water to compare its potency with that of PTU. In the 5th group, a prolonged hypothyroid induction was initiated by applying 0.05% MMI as soon as prenatal day 17 to postnatal day 26 to investigate the effect of exposure duration. Finally in the last group, a much lowered concentration at 0.0125% MMI was used to see whether the mild hypothyroidism can affect the expression of target genes examined in this study.

RNA preparation

Total RNAs were prepared by the Qiagen RNeasy mini-kits. Total RNAs were quantified by $UV_{260/280}$ measurement in a Gene Spec I spectrophotometer (Hitachi Genetic systems, CA.). Reverse transcriptions were performed using 50 μM poly dT_{12-18} as primer, 6 μg total RNA, 200 U SuperScript II RNase H$^-$ reverse transcriptase, 10 mM dNTP (Invitrogen Life Technologies, CA.) in DTT enriched 1X first strand buffer at 70°C for 10 min, 37°C for 1 h and an inactivation process at 90°C for 5 min using an DNA Engine (MJ Research Inc., USA). After all, the

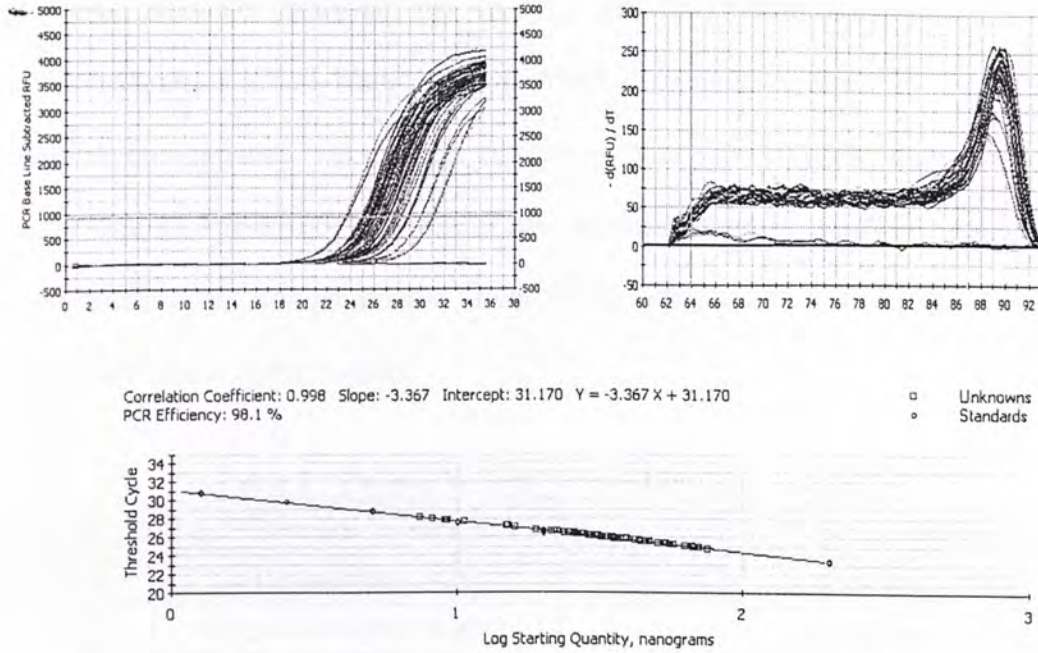
RT-products were cooled to 4°C, aliquoted to smaller portions and stored at -30°C until the real-time PCR processes.

RT-PCRs:

The RT-products were diluted to a concentration of 20 ng/µl for the subsequent PCRs. Equal amount of cDNA of all samples were pooled to form a large sample pool for the construction of standard curves for individual target gene in the following study. The conditions of the PCR such as Mg^{++} concentrations, annealing temperature were optimized before the RT-PCRs and real-time PCRs. The size of individual target gene was confirmed by running the PCR products in 2% agarose gel with 0.5 mg/ml ethidium bromide in 1X TAE buffer (40 mM Tris, 20 mM sodium acetate, 1mM EDTA, pH 7.2) with 50 b.p. or 100 b.p. DNA markers running parallel to the target gene samples.

Real Time PCRs:

For real-time PCRs, either the SYBR Green® core kit and the ABI PRISM™ 7700 sequence detection system (Applied Biosystem, CA) or a Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, CA.) based home-made PCRs and the Bio-Rad iCycler iQ® Real-time PCR system (Bio-Rad, CA) were used. Standard curves of target genes were made using the pooled RT products from all samples at the final concentrations of 0, 1.25, 2.5, 10, 40, 100 and 200ng. For each sample, a final concentration of 40ng of cDNA was subjected to PCR amplification in duplicates. A final reaction volume of 50 µl was used in all the Real Time PCRs with amplification environment optimized as in RT-PCRs. Melt-curves of each target gene in individual reaction wells were used to verify the production of single band in the individual well.



The amplification curve (top-left), melt curve (top-right) and the standard curve calibration (bottom) of the ADAM6 genes that were generated by the Bio-Rad iCycler iQ® Real-time PCR system. High correlation coefficient and PCR efficiency with single-peaked melt curve was ensured in each of the genes being studied.

Data manipulations and Statistics:

Results of the PCRs for individual target genes were normalized with β-actin as housekeeping gene. Expression of β-actin is tested in all samples not to be affected by age, treatment and their interactions in the experiment. The transcription of housekeeping β-actin in each individual samples (in ng) were divided by the median value of all the samples (also in ng) to obtain a normalization factor. The relative gene expression value of each individual sample is obtained by correcting the raw expression data (as calculated from the standard curve calibration; also in ng) with the normalization factor. All data were subjected to statistical calculation by 2-way analysis of variance (ANOVA) using the statistical package SigmaStat 2.03 for Window (SPSS Inc., IL). In case the collapse of one factor in the analysis, either

one-way ANOVA followed by Tukey's test for multiple comparisons or the non-parametric Kruskal-Wallis one way ANOVA on ranks followed by Dunn's test for multiple comparisons was used for comparing data pairs (Illustrated as number-pairs in the figure). For the comparisons of two samples either the unpaired Student's t-test (two-tailed) or the non-parametric Mann-Whitney Rank Sum test was used (Illustrated as alphabet-pairs in the figure).

Table 1: Primer sequences used in this experiment:

Gene	Sense sequence Antisense sequence	Gene	Sense sequence Antisense sequence
α_2MG	ACTGGAAGCCGTTCTGAATCC TGGCTTCAGGTCTCTTATTGG	3β-HSD	TGCAGACAAAGGCCAAGG ACACTGGCTTCGACGCAGG
ABP	TGATTGCTTCTCAGAAGAGGC AGAGCTGCATTCAGGAAAGC	ACE	CAGCTTCATCATCCAGTTCC ACATACCCAGGAAGTTGACG
Activin βA	CTGGAAAAACGAGTCATCTGC GCATTACCTTTCGGGTCCCT	Act	TGCCTGGATTGCTACAACC TTTCTGGGTCAAGAATCCTTCC
ADAM3	GGCCCAGCAGAGAGATGTTC CACTGGTCCGTACTTTTCCTA	ADAM2	CATGCTGTAATCCCACCACC TACCCCGTAGGAGTGATACC
ADAM5	TACCTGGCCATTCAAGAGG ACTGACTCACAATAGGGAGG	ADAM4	GGTACTTTAAAAGACTGTCTC CAAATGCCATTCGTGTGCCT
ADAM10	TCTGACTGTGCAAAGGAAGG TAGCTAGAGGGCCATCAGC	ADAM6	GGCTCACATAAGGCATGTTAC ATATGTTCTGGATCGGTCTGC
ADAM17	TCTGCGGCTCCAAAACTGG TGACGCTGCAGCTTGAAGG	ADAM15	GGCTTCCAGATGAGTGCAC CAATCGTCTGTGGGAAGGCG
ADAM30	TGCCAGGCCATTTTTGGACG GTACCGTCGTTGACATCACC	ADAM18	GGTAACGAGACGGAATGTCAA ACTCTGGGTCAACAGCCTTC
ADAMTS-1	CATCCAGGTTCTTATGGTGG CATGGGACAGACATTCAAGG	ADAM32	TCTGCATCATGAATCCCAGG TCCATACATAGCCTCGCATGC
ADAMTS-5	ATGACCATGAGGAGCACTACGA GGAGAACATATGGTCCCAACGT	ADAMTS-4	GACCTTCCGTGAAGAGCAGTGT CCTGGCAGGTGAGTTTGCAC
Axin	ACCACCATGTGACAGTATCG TCCCGTACTTCCTCAAATACC	ADAMTS-16	TAAGTACCGCAGCTGCACGG CAGCAAGGCATATTGCTTGG
β-actin	TCATGAAGTGTGACGTTGAC CCTAGAAGCATTTGCGGTGC	β_2MG	GTGTCTCAGTTCCACCCACC AAGTGACGGTTTTGGGCTCC

Gene	Sense sequence Antisense sequence	Gene	Sense sequence Antisense sequence
Cadherin-8	TTTGGAACTAAGCAGGCAGAGTGAA TCACCCGATAGGATATACTTGATTT	Bikunin	AGGAATGCCTGCAGACGTGC AGCTCCTCGTACCCATCACC
Calpain I	GAAGCGTCCTACGGAACTGCTG GCATAGGCTTTCTCCAGGAGAGC	Cadherin-11	CCTGACGGTATCAATGGATTTA GGTCCCCAGTTCTGTAGATAGT
Cathepsin B	GAAGCCGGTGATGTGATGGGAGGC CAACCTTGCTGAAGCTTCATGC	Calpastatin	AGTCTTGGACAAAGACAGC AGTCTTCTTACCCTTCTCC
Cathepsin L	GAACGCCTTCGGTGACA CCTGATTGCCTTGATCGTGAGA	Cathepsin C	TTAGCCCTGTTCGAAACCAAGAAT CTCAGTCCAGTGTGGTGGTAGATC
cdc42	CACCACTGTCCAAAGACTCC CACTCCACATACTTGACAGC	CD44	TATCATCTTGGCATCCCTCC ATCCACACTCTGCAGATTCC
Claudin-11	CTGTATCCGAATGGGCCACG TAGACATGGGCACTCTTGGC	Claudin-1	TATTAGTGGCCACAGCATGG TCCCACTAGAAGGTGTTGGC
Col3α1	AGTTCAGCTATGGCAATCC TCACCAGTGTGTTTAGTGC	Clgn	AGATTGGATGTGGAGAATGG AGATATCAGGAGTCATGGACC
Cx43	ACAGCTTGTTGAGTCAGCTTG ACGAATATGATCTGAAGGAC	CREMδ	ACTACTGCTTTGCCACAAGG TTTTCAAGCACAGCCACACG
Cystatin C	AACGATGCGTACCACAGC TGCAGCTGGATTTTGTCAGG	Cystatin 8	GTGAGGACAAATACCTCTTCC TGCTCCTACCAGAAAGCTGC
Cystatin TE-1	TTAGTAGACCTGGAGATGGG ACAGGTGCTGTTCAAGATGG	Cystatin SC	TTGCTAGAAGTTGGGAGAGC AAGTGGGAAAACCAAGGTCG
E-Cadherin	TGAAGACTCCCGATTCAAAGT TTGATTTTCCGGGCAGTTGAT	Dncic1	TCGTCATGGAAGCAAAGC AGTCACCTACTGCAACTTCC
GATA-4	CAGCAGCAGTGAAGAGATGC AGAGTCCTGCTTGGAGCTGG	Eppin	AAGATACTGGCCTCTGTACC TTCTCGTTGCCTCTGCAGC
KRP2	ATGATCGCCATGATCTCACC ATCCGTCATCTCAGAGAGC	Id-2	AGCCTTCAGTCCGGTGAGGTC TCAGACGCCTGCAAGGACAGG
MGP	AGAGATGGCACGCTAAAGC TGAAGTAGCGGTTGTAGG	LDHc	TGAAGGGCTATACCTCTTGG TCTGTGATGCCACTTTGTCC
MT3-MMP	ATCACCTCAGGGAGCTTTCG CATAAGGCCAAGATGCAGGG	MMP-9	CGTGGCCTACGTGACCTATG GCATAGCTCGGTGGTGTCCT
Nedd 4a	TCAGTTTGTTACTGGCACATCC ATCCCAGAGTTCGTCAAAGG	N-Cadherin	GCCACCATATGACTCCCTCTTAGT CAGAAAACTAATTCCAATCTGAAA
Nexin-1	AAGGTCAGAGAGCCTTCACG TCGGATGCAGAACAGGAAAGG	Nep	TTGGGAACTAGCAGACAACC AGCACCTTAAGAGGACTGTGG

Gene	Sense sequence Antisense sequence	Gene	Sense sequence Antisense sequence
Occludin	GCAAAGGGTTTCCTGCTGGC CGTACATAGATCCAGAAGCC	NT3	GCTATGCAGAGCATAAGAGT CAGATGCCAATTCATGTTCT
PACAP38	GTACCTGCAGTCCATGGTGG AAGTACGCTATTCGGCGTCC	Odf1	TGAGTGCCATTTCCCAAAGG TGGAGTAGCAGAGGCAGTAGG
PAI-1	TCTTTCCGACCAAGAGCAGC CTGTTGGATTGTGCCGAACC	PACAPr	TGGTGGCTGTACTCTACTGC GCTGCTCTTGCTCAGGATGG
Pcsk4	TCATGTCTACTCACTACTGG AATGACTTTCCTGGCACAGC	PCI	ACCCATGCTGACTTGTCTGG TCAGGGCTGAATCACTTTGC
Prm 2	TGGAGGACTATGGGAGG TCCTTCGGGATCTTCTGC	Pki β	TCTGTAATCAGCAGCTTCGC AGAGGTTACCATGGGTTACC
Ras GRP-1	TGCACAGTCCAGCATCTCCG AAGTACCATCACCGTGGTCC	PRSS21	TGCTTTGGTGACTCAGGAGG AGGCCAGAGTAAGAAACAGC
Rho A	TCTGAGGCCTCTCTCCTACC TTGCAGAGCAGCTCTCGTGG	Rhes	AGGAGACGTCTTCATCCTGG ACTGCACGGAGATCTTGTGG
Sert-1	AGAAGACAGCAATGGTTGGG AGGATTCAATACGAAGCAGC	sACE	AACAGGTGCTGCAGGCTGG TGCCAGTTGGCCTCTGCGT
Sp10	AGTACACCACTTCCAAGC AGACTTCATGACGACAGC	Sertolin	GCTATGAGGGTCACCATTGG AAACGACCCTGAACAGCTCC
Spam	AGTCAATGTCACCCTAGCAGC TTTGCCAGGCGTTGAACTGC	Spink2	TGCTGAGACTGGTGCTGTTGC TCAGCATGGCTCGTCCTTG
Syndecan-4	AGGAACTGGAAGAGAATGAGG TCTTGCCCAAGTCGTAACTGC	Sycp1	GAACAGGAACAGTCTTCAGC TTCCAACTAGTGGCTTCAGG
Testin	TGGTTCCTTCCAGTTCTACG AGTGGTTGCTCCAGTCTTTGG	tACE	GGCTACTCCAGGACTGCC TGCCAGTTGGCCTCTGCGT
TIMP2	TGCGAGTGCAAGATCACACG TCGATGTCAAGAAACTCCTGC	TIMP1	GCAAAAGGCCTTCGTAAAGACC AGGTGCACAAATCTGGATTCC
TIMP4	AGAGCCTGAATCATCACTACC TGCAGATGCCATCAACATGC	TIMP3	CGACATGCTCTCCAATTTCG TCAGGGGTCTGTGGCATGC
TP2	AGGTGAGCAAGAGAAAGG CCTAGTGATGGCTATCTCC	TP1	AGCCGCAAACTAAAGACTCATGG TCATTGCCGCATTACAAGTGG
TRα1	CGACGCCATCTTTGAACTGG AACTCTGCACTTCTCTCTCC	tPA	ACGGGACACGGAAGAAACGG CCCTCGAAGCAGGTTGCTCTG
Zfp37	TCAGAAGTCACACCTCATCG AGGGTTTATAGGGTTTCTCTCC	uPA	TCACGAACAGTGCAAGCAGC TTTCTCTGCACATCCACTGC

Results:

Body and testis weights, together with the transcription profiles of each gene are shown in the following paragraphs. Unless specified, solid black bars represent the data in control rats, while solid grey columns denote that of the PTU-treated rats suffered from neonatal hypothyroidism. X-axis showing the age of rats being scarified is not in a linear scale. Vertical bars with ticks represent the arithmetic mean ± standard error of mean within each group. Numbers on top of the columns show statistical significance between the data pair by multiple comparisons. Paired alphabets show statistical significance by unpaired Students' t-test or Mann-Whitney Rank Sum test. For simplicity, * represents $p<0.05$, ** stands for $p<0.01$ and *** denotes for $p<0.001$ for significantly greater values during pair-wise comparisons. Screening data is also attached for relevant discussions.

Effect of neonatal hypothyroidism on the developmental profile of body weight, absolute and relative testis weight:

Body Weight

As shown in Fig 1.1, hypothyroid rats are all significantly lighter than controls. Drastic reduction of 50% off the total body weight was found from day 30 to day 50. Hypothyroid rats still suffered an 18% decreased in size, and are not able to recover back to normal until day 90. All data pairs comparing the body weight of normal and hypothyroid rats are significantly differed.

Absolute Testicular weight

As illustrated in Fig 1.2, the absolute testicular weight is always significantly smaller in hypothyroid rats until day 60, where a smaller decrease was noticed. Ultimately the testis sizes in hypothyroid rats were 40% larger than normal at day 90.

Relative Testicular weight

In Fig 1.3, the relative testicular weight, which is the division of absolute testicular weight over total body weight, revealed a developmental change. Though a 40% decrease was profound between day 24 to 40 (* and ** stand for $p<0.05$ and <0.01 respectively), drastic rebound can be illustrated after the age of sexual maturation after day 50 (** and *** represents $p<0.01$ and <0.001 respectively).

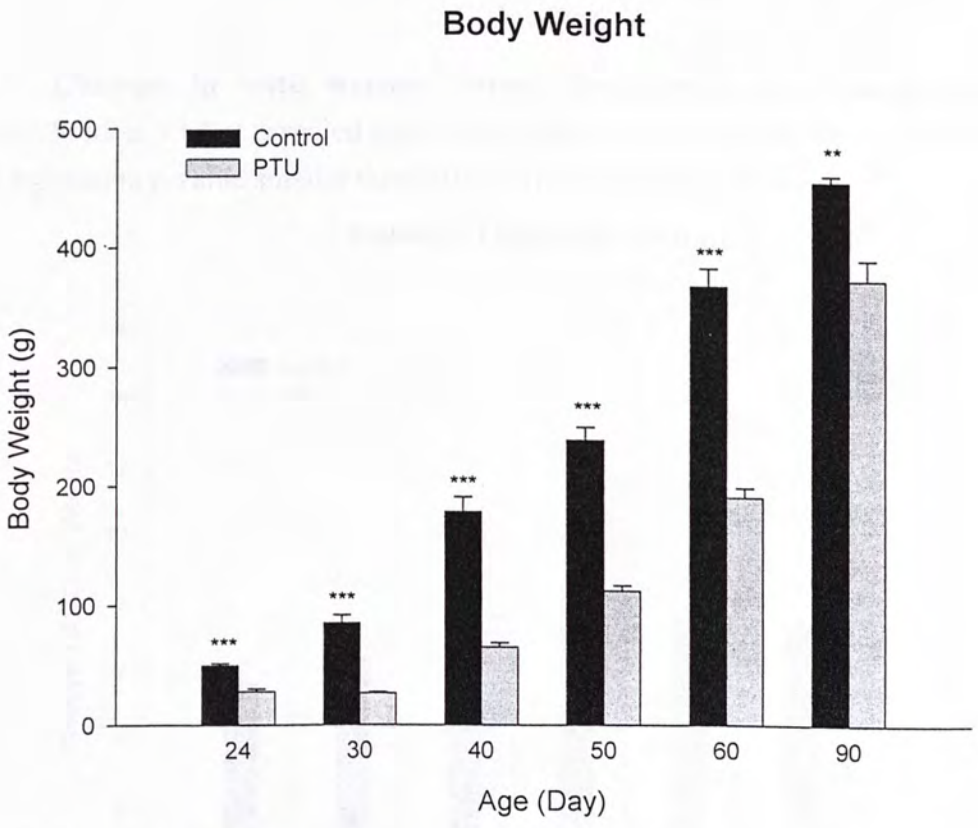


Fig 1.1 Changes of total body weight during development. ** and *** denote p-value smaller than 0.01 and 0.001 respectively using unpaired Student's t-test (Two-tailed).

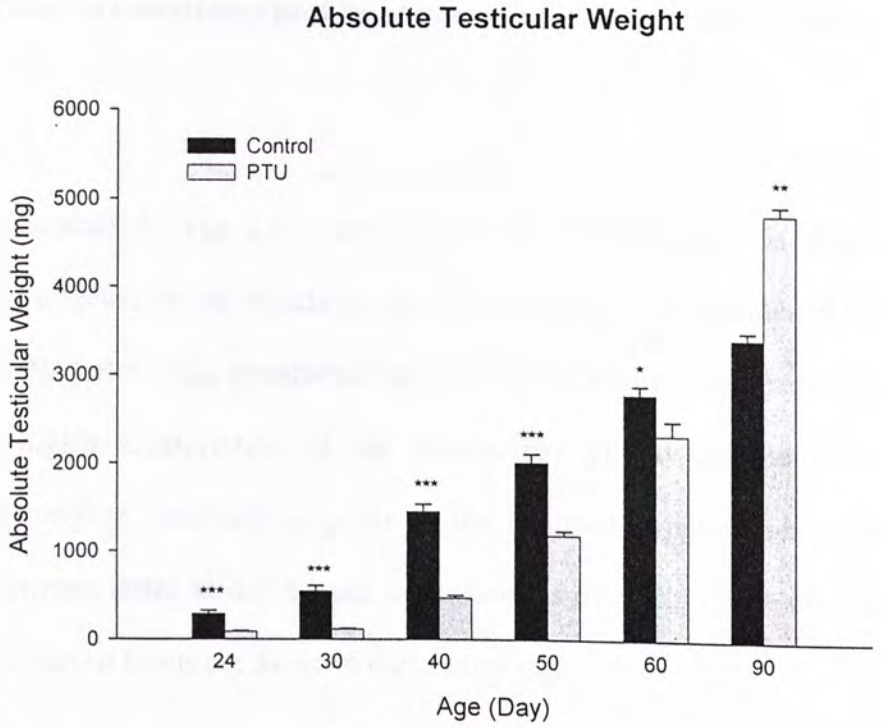


Fig 1.2 Changes in testis weights during development (per paired testes). Unpaired Student's t-test revealed significant differences as denoted by *, ** and ***, which represents p-value smaller than 0.05, 0.01 and 0.001 respectively.

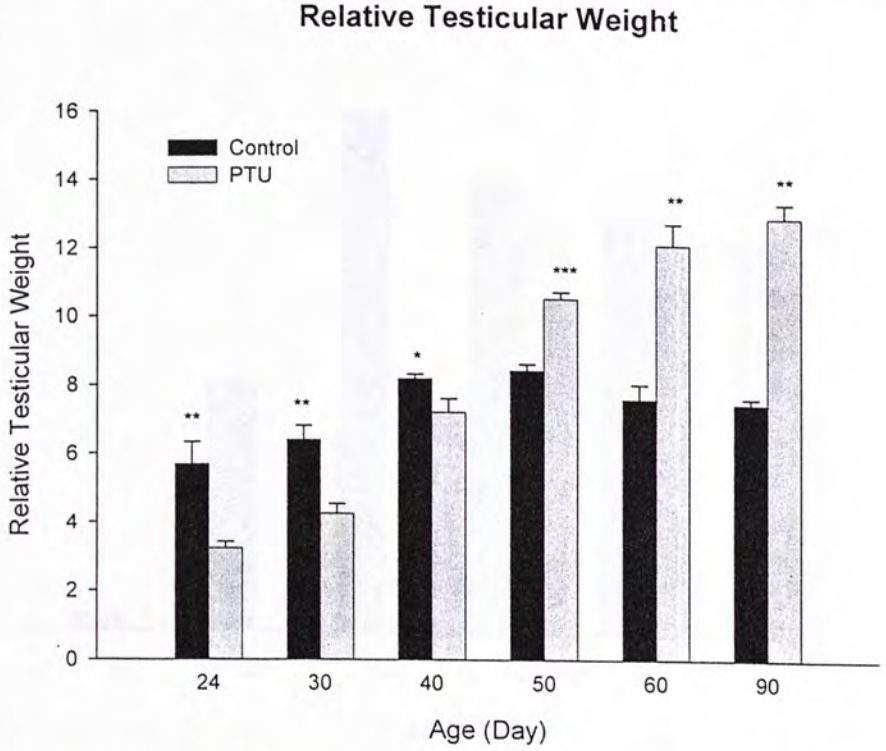


Fig 1.3 Changes in relative testicular weight during development (per paired testes). * represents $p<0.05$, ** stands for $p<0.01$ and *** denotes for $p<0.001$ for significantly greater values during pair-wise comparisons.

Developmental transcription profiles of genes under normal and hypothyroidism

ADAM2:

As illustrated in Fig 2.1, transcription of ADAM2 gene is found to be significantly affected by the effects of age, treatment and their interaction (results of 2-way ANOVA after $\log_{10}$ transformation; $p<0.001$). Multiple comparisons indicate significant higher transcription of the control rats at day 40 than at day 24. Significantly higher transcription levels in the neonatal hypothyroid rat testis are observed between testes at day 60 and 24 as well as day 30. At day 40, significant higher transcription levels are found in the control rats.

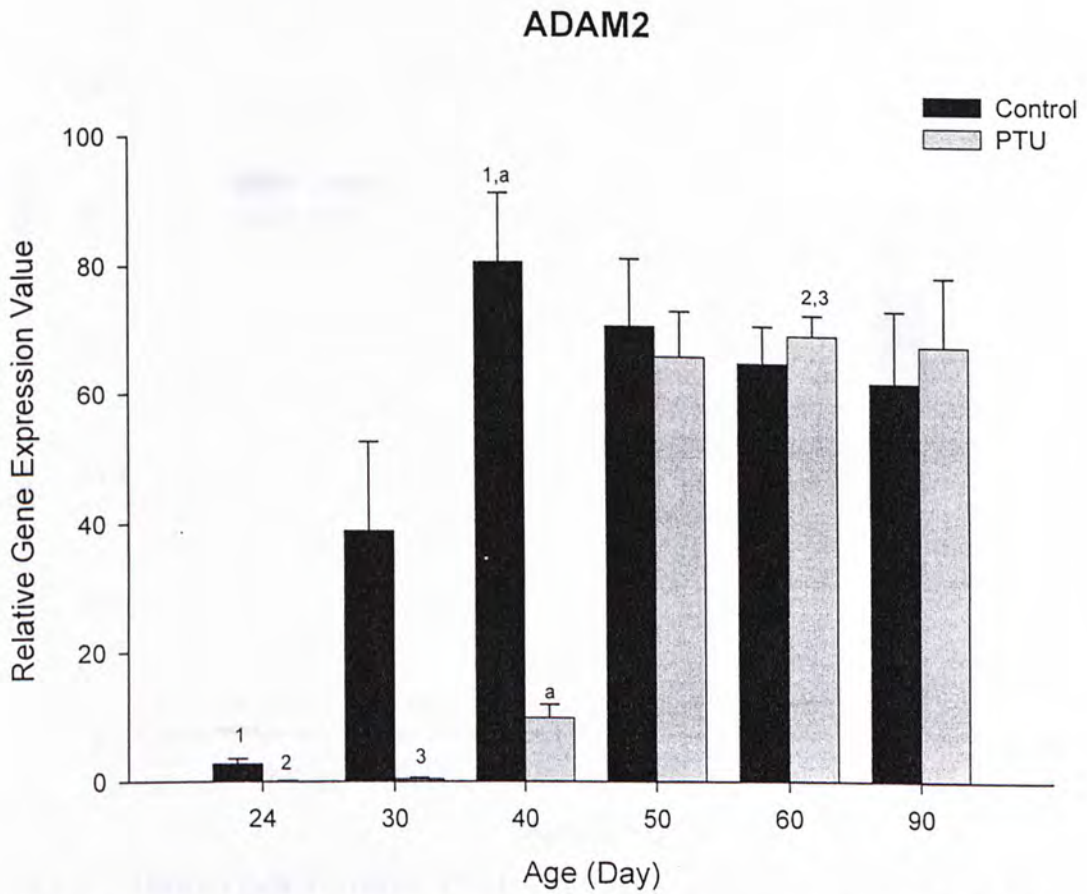


Fig 2.1 Transcription Profile of ADAM2 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. "a" shows statistical significance by unpaired Student's t-test with $p<0.01$.

ADAM3:

As shown in Fig 2.2, transcription of ADAM 3 gene is significantly affected by age ($p<0.001$) and treatment ($p<0.01$) but not by their interaction [results of 2-way ANOVA after square root transformation]. Multiple comparisons indicated that between the control rats at day 24 and 90 and the neonatal hypothyroid rats at day 24 and 60; 30 and 60 as well as day 24 and 90; 30 and 90, significant higher transcription levels are observed in the older rats. Using pair-wise comparison to evaluate the control and neonatal hypothyroid rats at day 24 and 30, significantly higher transcription is observed in the control.

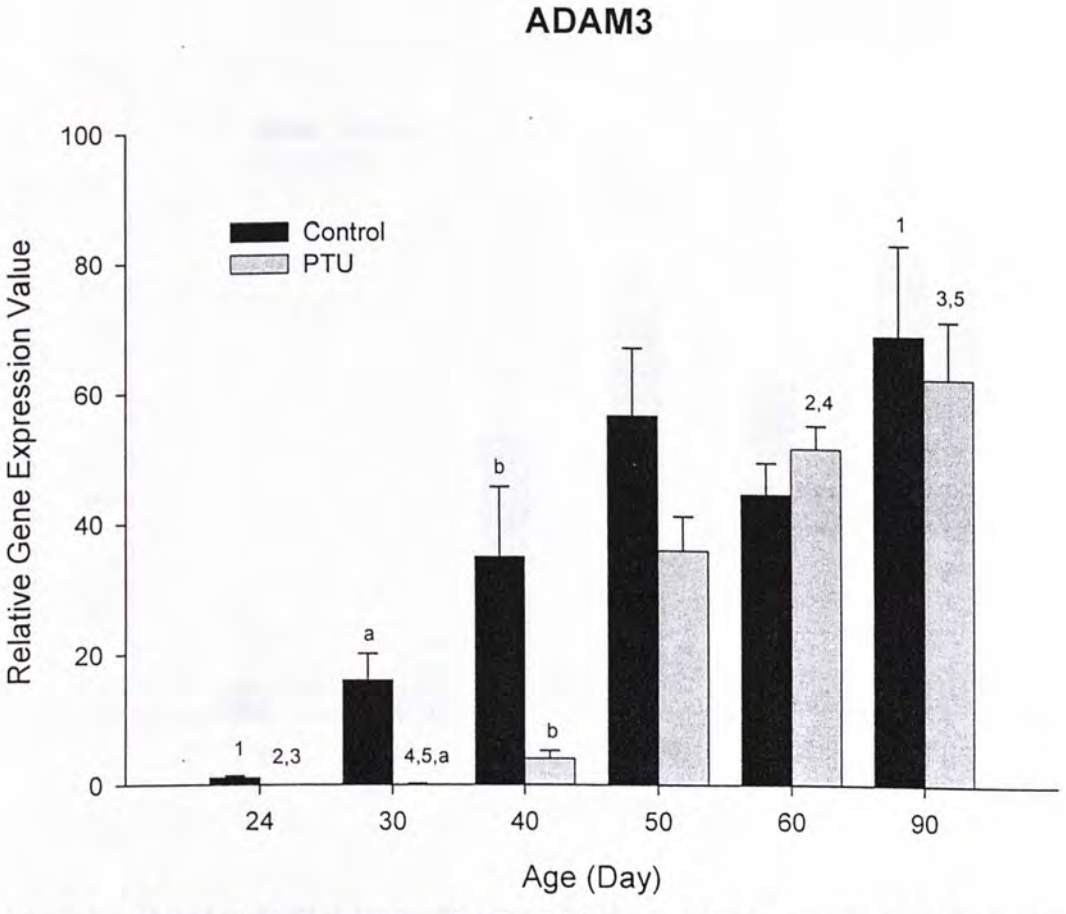


Fig 2.2 Transcription profile of ADAM3 gene. Numbers on top of the columns show statistical significance between all the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by Mann-Whitney Rank Sum test, a: $p<0.01$, b: $p<0.05$.

ADAM4:

As indicated in Fig 2.3, transcription of ADAM4 gene is significantly affected by age, treatment and their interaction (results of 2-way ANOVA after $\log_{10}$ transformation: $p<0.001$). Transcription of this gene in the control rats reaches the adult's levels at day 40, while the catch-up of the neonatal hypothyroid rats starts at day 60. At day 40 and 50, control rats have higher transcription levels than the neonatal hypothyroid rats.

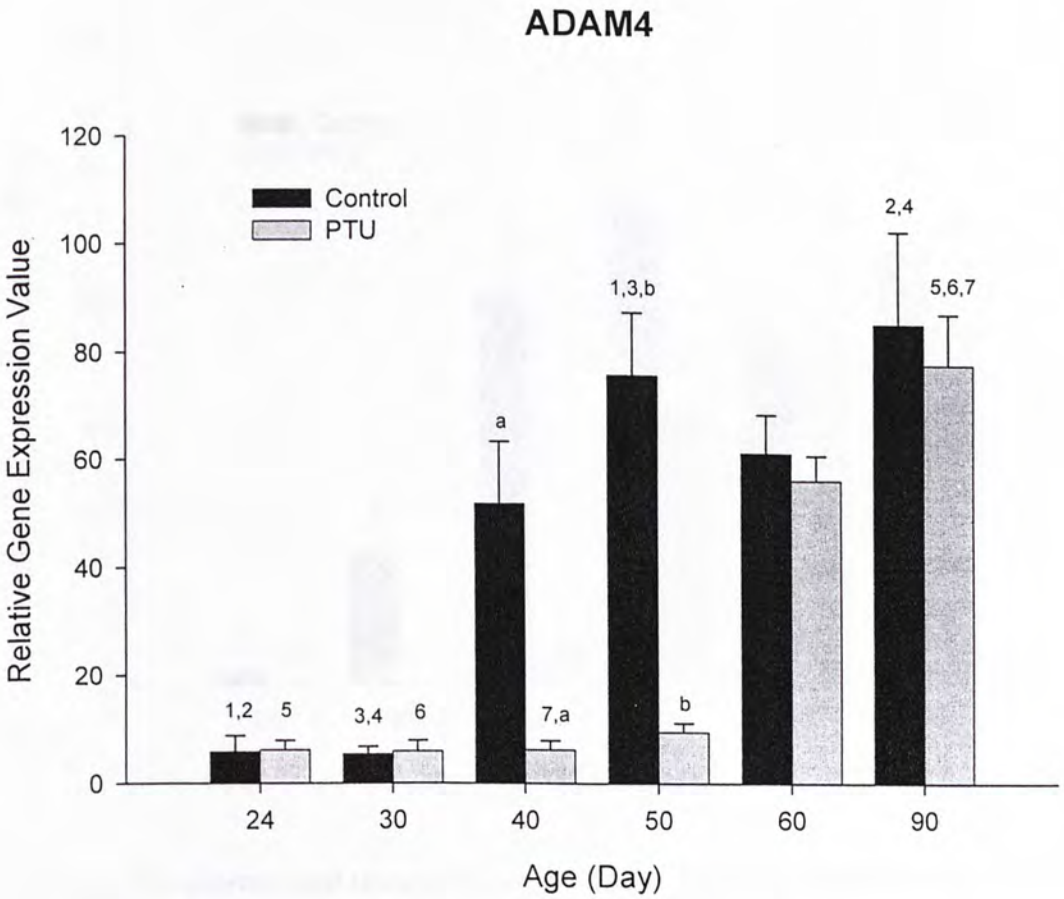


Fig 2.3 Developmental transcription profile of ADAM4 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison, a: unpaired t-test with $p<0.01$, b: W-M Rank Sum test with $p<0.01$.

ADAM5:

As illustrated in Fig 2.4, significantly higher transcriptional level is observed in the control (Day 50 vs day 24) and the neonatal hypothyroid rats at advanced ages (Day 60 and 90 vs day 24 and day 30). Control rats have higher transcription levels than the neonatal hypothyroid rats from day 24 to 50.

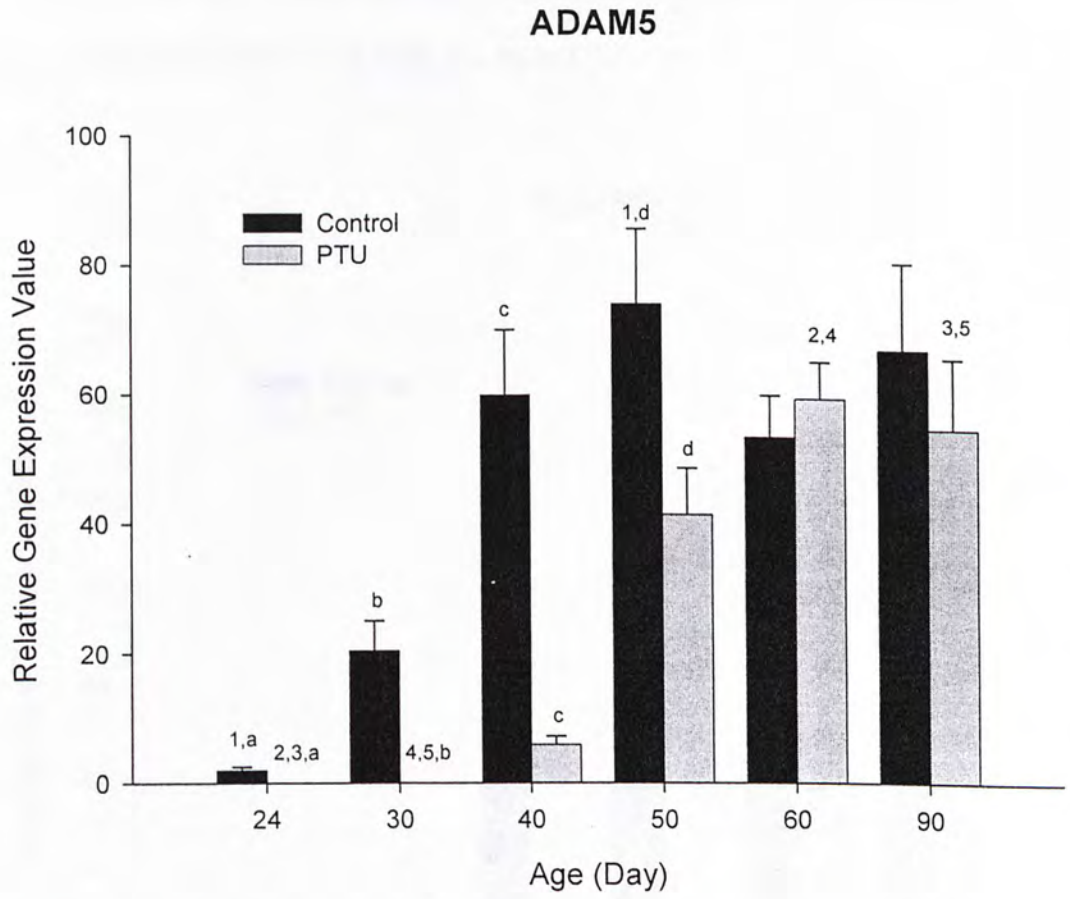


Fig 2.4 Developmental transcription profile of ADAM5 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison. a: Rank Sum test with $p<0.01$. b,c,d: Unpaired t-test with $p<0.01$, <0.001 and <0.05 respectively.

ĂDAM6:

As shown in Fig 2.5, the transcription of ADAM6 is very similar with ADAM 4. Results of two-way ANOVA after square root transformation show that age, treatment and their interaction significantly affected the transcription of this gene ($p<0.001$). Older rats use to have higher transcription levels than the younger rats. This is true for both control rats (Day 50 & 90 vs 24 & 30) and the neonatal hypothyroid rats (Day 90 vs 24, 30 and 40). Control rats have higher transcription levels of this gene than the neonatal hypothyroid rats at both day 40 and day 50.

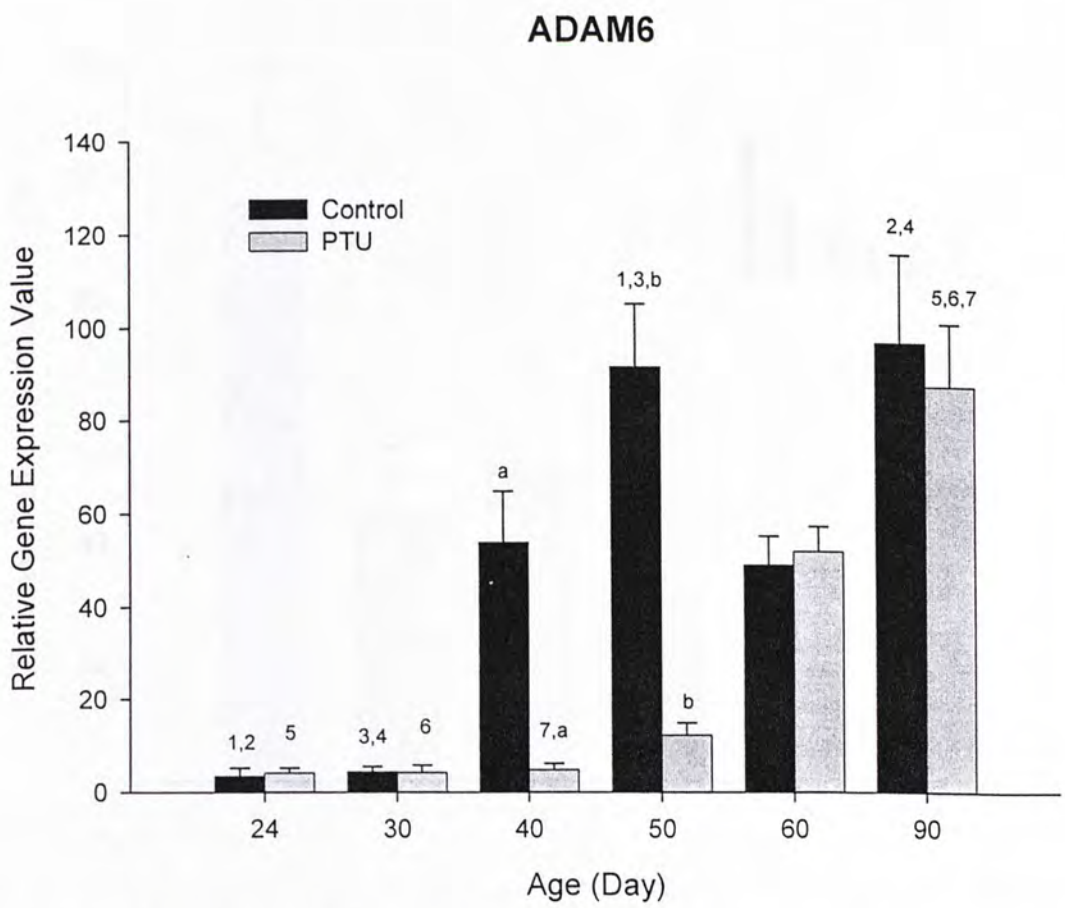


Fig 2.5 Developmental transcription profile of ADAM6 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Unpaired-test with $p<0.01$. b: Rank Sum test with $p<0.01$.

ADAM15:

As illustrated in Fig 2.6, only a significant age effect on the transcription of ADAM 15 is profound (Results of 2-way ANOVA after log10 transformation: $p<0.001$). A gradual drop of its transcription in the developing rat testis is illustrated. Higher transcription levels are usually observed in the younger animals.

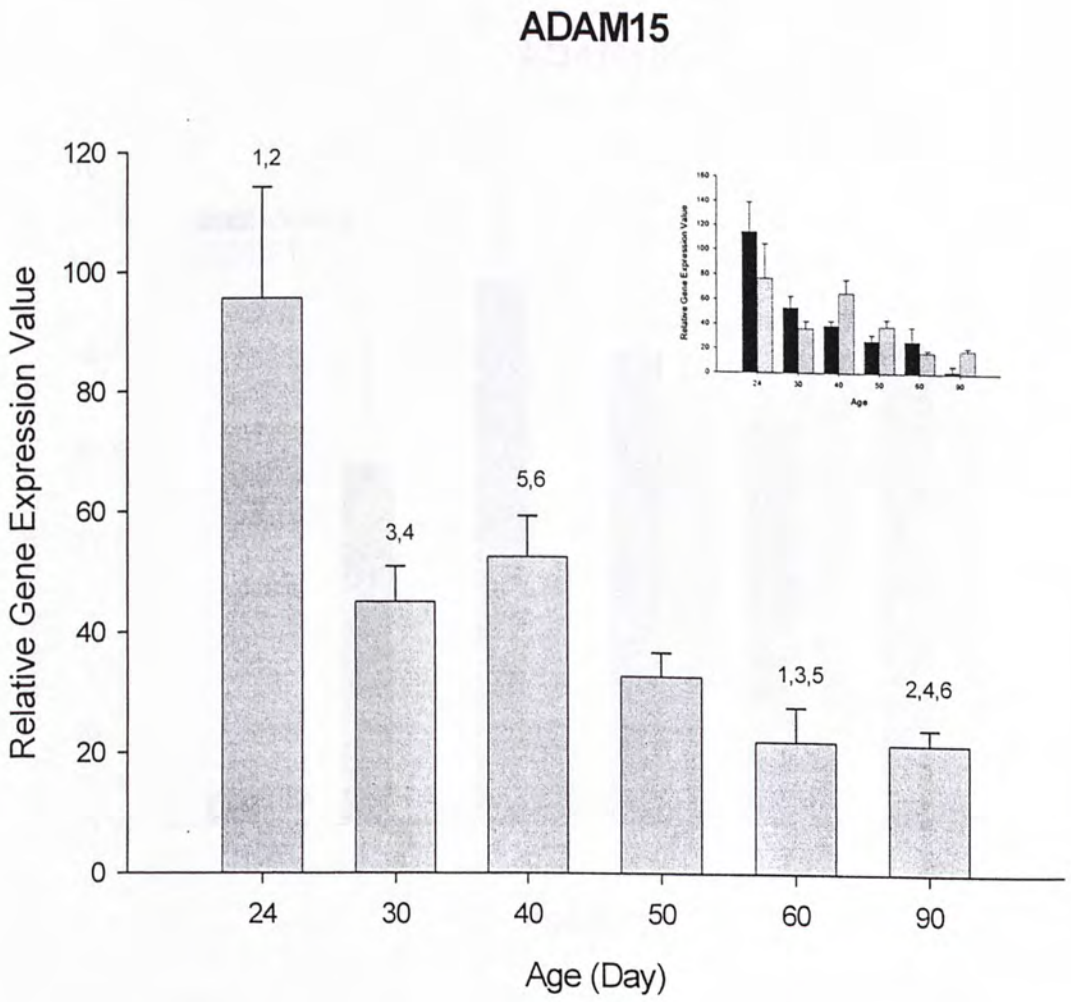


Fig 2.6 Developmental transcription profile of ADAM15 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test after ANOVA by Rank with all $p<0.05$.

ADAM18:

As shown in Fig 2.7, maturation of the transcription of this gene in the rat testis is some how earlier than other genes in this study. All the factors examined with two-way ANOVA after $\log_{10}$ transformation: $p<0.001$. In the neonatal hypothyroid rats, rats at day 60 have higher transcription levels than those at day 24 and 30. Significant higher transcription levels are also observed in the control rats than in the neonatal hypothyroid rats at day 24 and 30 as well as at day 40.

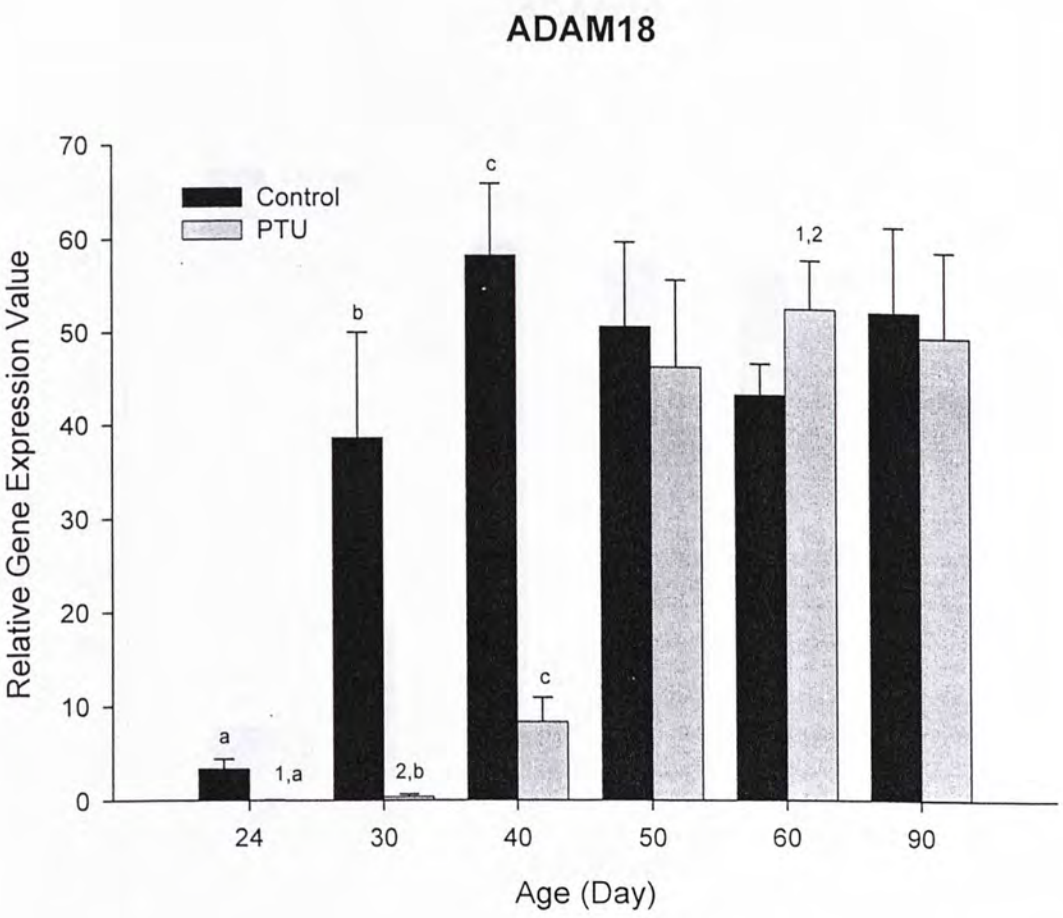


Fig 2.7 Developmental transcription profile of ADAM18 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison. a, b: Rank Sum test with $p<0.01$. c: Unpaired t-test with $p<0.001$.

ADAM30:

As shown in Fig 2.8, transcription of ADAM 30 gene is similar to those of ADAM2 gene. Results of two-way ANOVA after square root transformation show that age, treatment and their interaction significantly affected the transcription of this gene ($p<0.001$). Recovery of transcription of this gene in the neonatal hypothyroid rats is observed at day 50. Transcription of this gene is higher in the advanced ages in both control (Day 40, 50, 60 vs 24) and the neonatal hypothyroid rats (Day 60 vs 24 & 30).

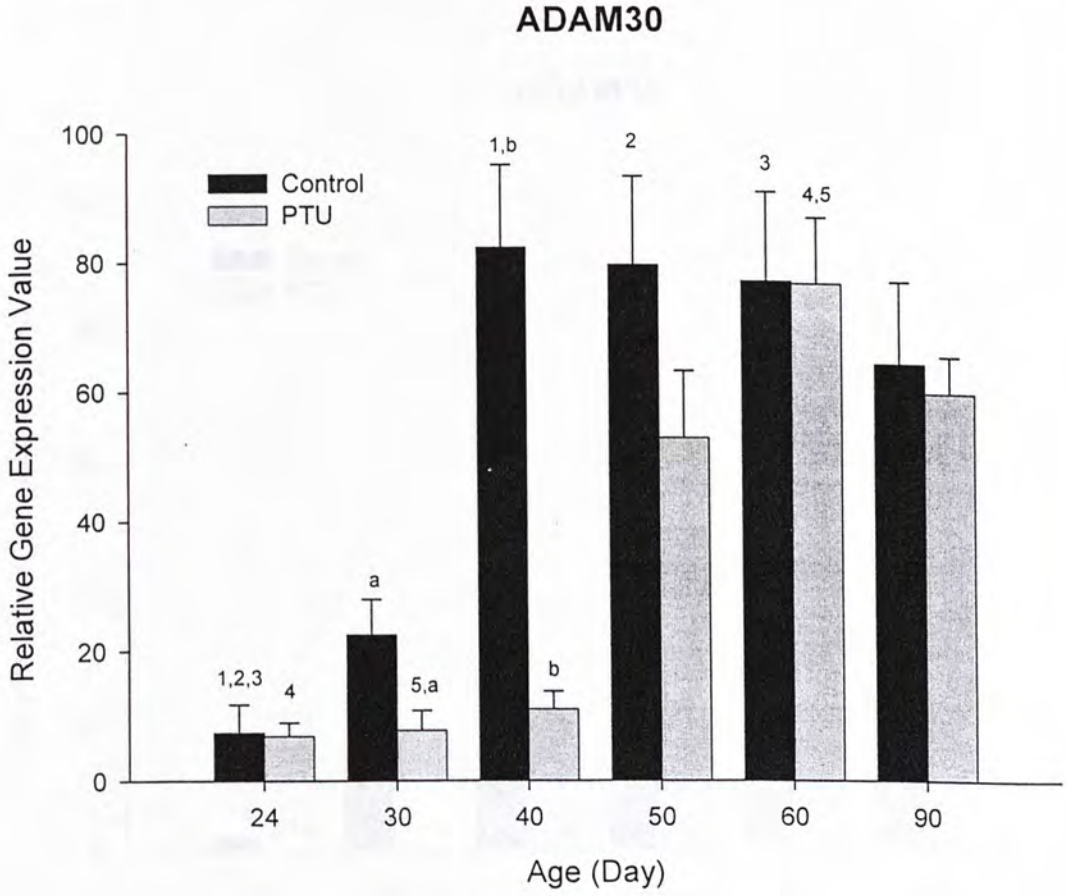


Fig 2.8 Developmental transcription profile of ADAM30 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison. a, b: Unpaired t-test with $p<0.05$ and $p<0.001$ respectively.

ADAM32:

Very similar to the transcription profiling of ADAM30, all the factors examined with two-way ANOVA after log10 transformation are highly significant at the level of $p<0.001$ as shown in Fig 2.9. Control rats at day 40 have significant higher transcription levels those at day 24. Significant higher transcription levels are also observed in the neonatal hypothyroid rats at day 60 and 90 than those at day 24 and 30. Significant higher transcription levels are also observed in the control rats than in the neonatal hypothyroid rats at day 24 and 30 as well as at day 40.

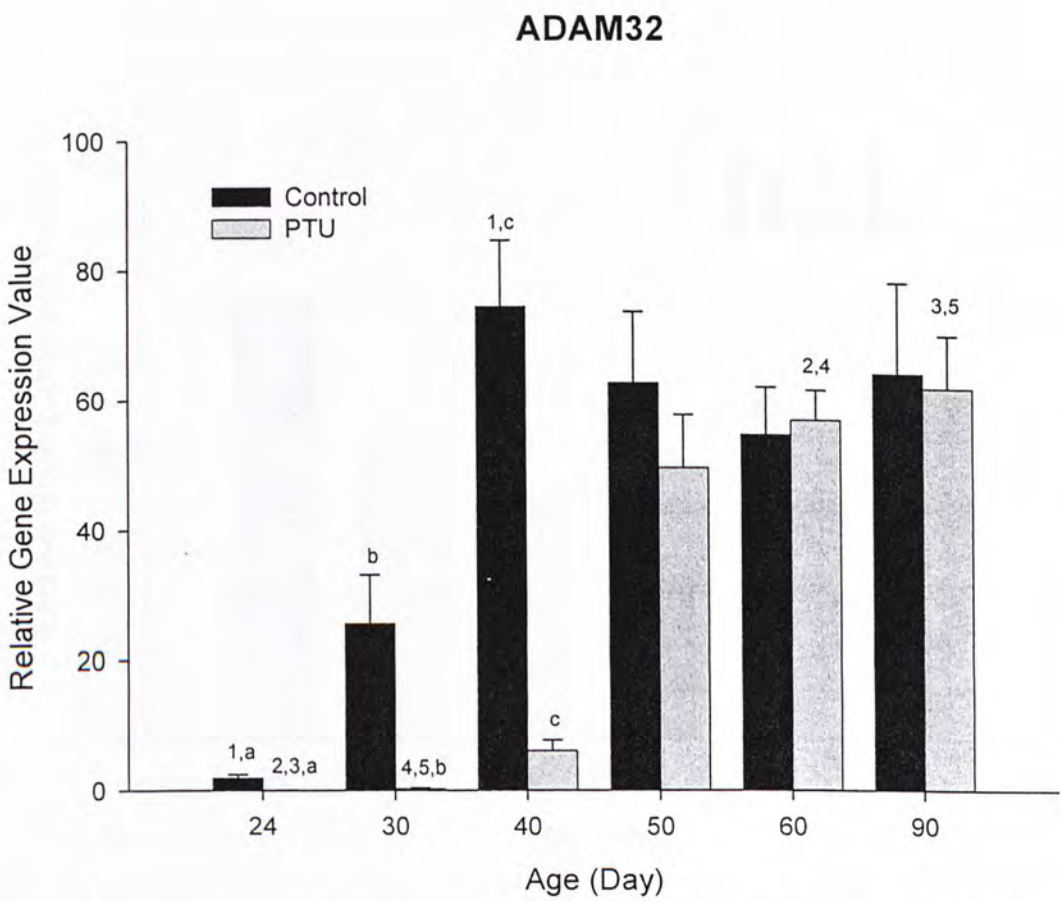


Fig 2.9 Developmental transcription profile of ADAM32 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison. a, b: Rank Sum test with both $p<0.01$. c: Unpaired t-test with $p<0.001$.

ADAMTS-1:

As illustrated in Fig 2.10, only a significant age effect is found in the transcription of this gene (two-way ANOVA after log10 transformation: $p<0.001$). Multiple comparisons with Dunn's test after ANOVA by Ranks reveal significant higher transcription of this gene at day 24, and 30 than at day 90.

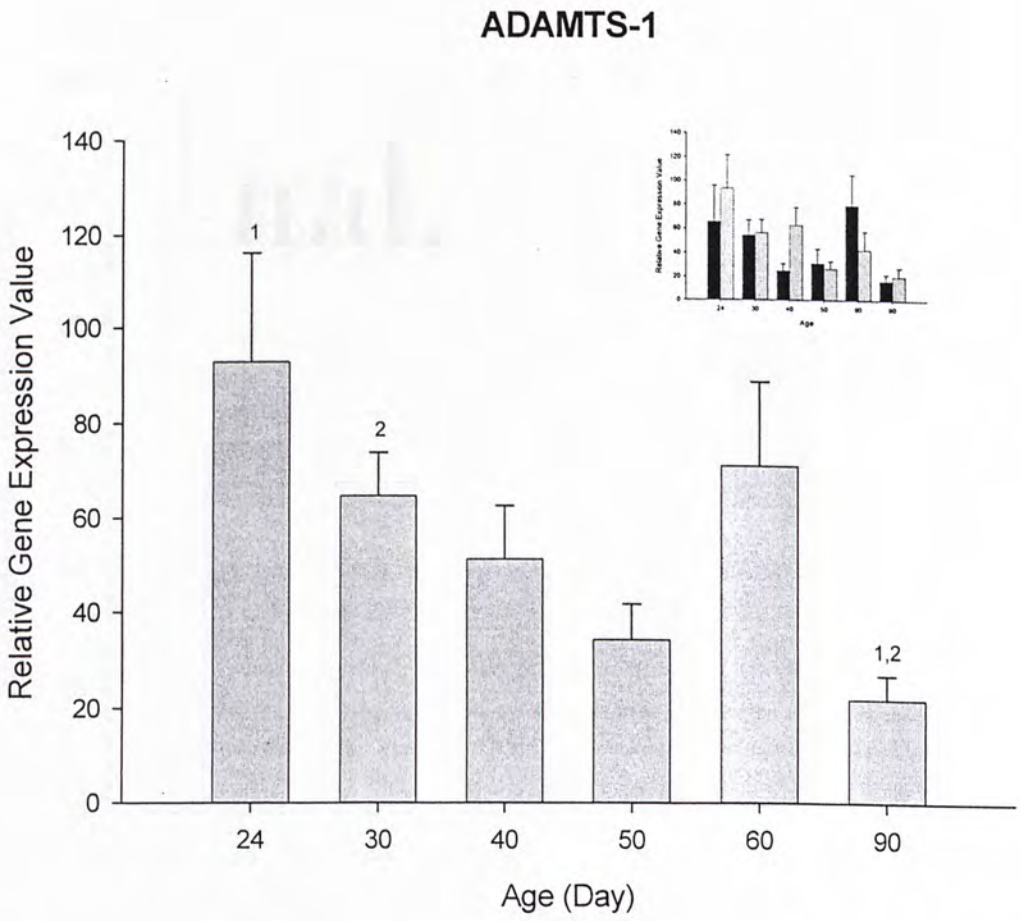


Fig 2.10 Developmental transcription profile of ADAMTS-1 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison. a, b: Rank Sum test with both $p<0.01$. c: Unpaired Student's t-test with $p<0.001$.

ADAMTS-4:

As illustrated in Fig 2.11, the pattern of transcription of ADAMTS-4 gene is similar to ADAMTS-1 gene. Only an age effect is significant (Results of two-way ANOVA after $\log_{10}$ transformation: $p<0.01$). Multiple comparisons with Dunn's test reveal a significant difference between the day 60 and 90 rats.

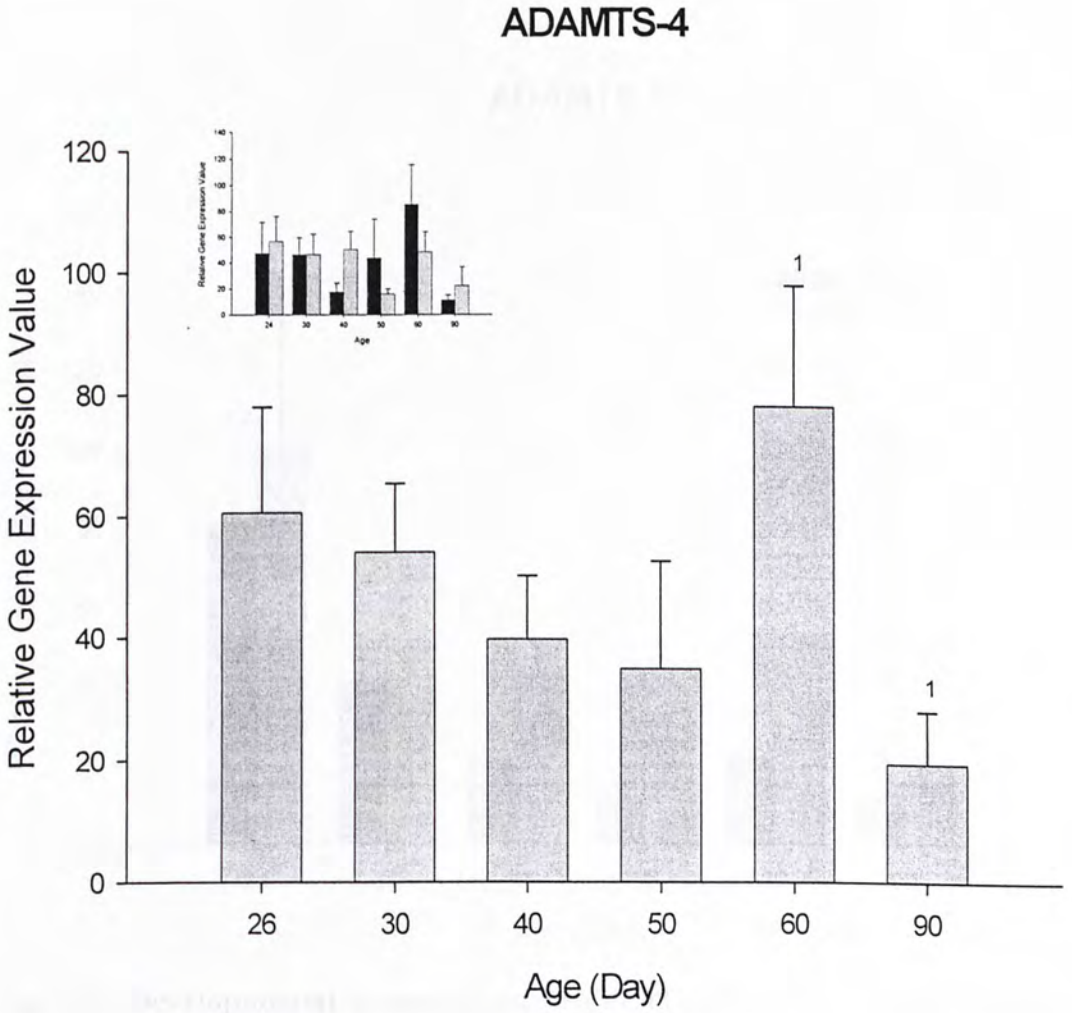


Fig 2.11 Developmental transcription profile of ADAMTS-4 gene. Number on top of the columns show statistical significance between the data pairs by Dunn's test after ANOVA by Rank with $p<0.05$.

ADAMTS-5:

Significant age ($p<0.001$) and treatment ($p<0.01$) effects are found in the transcription of ADAMTS-5 gene (Two-way ANOVA) as shown in Fig 2.12. A progressive decrease of its transcription is evidenced with significant higher transcription levels at day 24 than those of day 50 and 90 control rats. Significant higher transcription levels of the neonatal hypothyroid rats is only found at day 40.

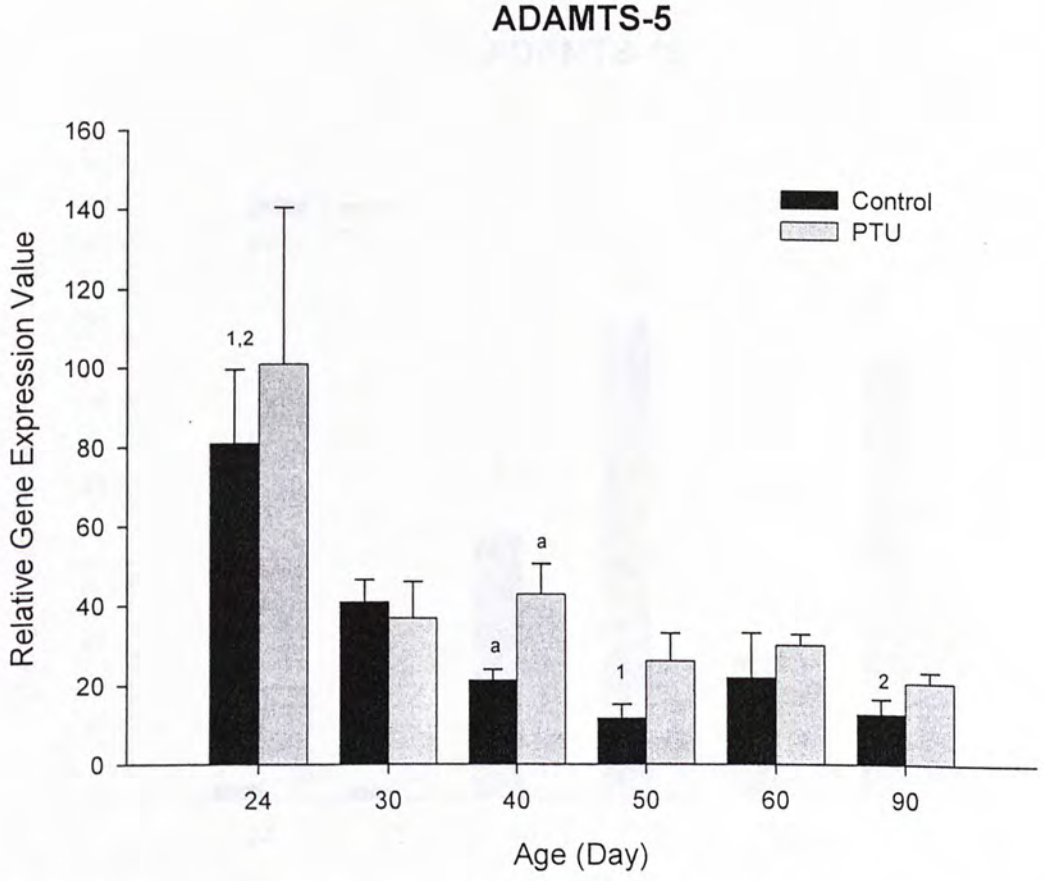


Fig 2.12 Developmental transcription profile of ADAMTS-5 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Unpaired t-test with $p<0.05$.

ADAMTS-16:

From Fig 2.13, the transcription of ADAMTS-16 is different to the patterns mentioned earlier in this study. Effect of age, treatment and their interaction are all significantly affecting of this gene (Two-way ANOVA after $\log_{10}$ transformation: $p<0.001$). Comparisons with Dunn's test reveal significant higher transcription levels in day 50 vs day 24 and 30 as well as day 90 vs day 30 control rats. Transcription of this gene in neonatal hypothyroid rats at day 90 is significantly higher than at day 40. Control rats have higher transcription than the experimental rats at day 40. At day 24, transcription of this gene is significant higher in the experimental than the control animals, while the opposite is true at day 50.

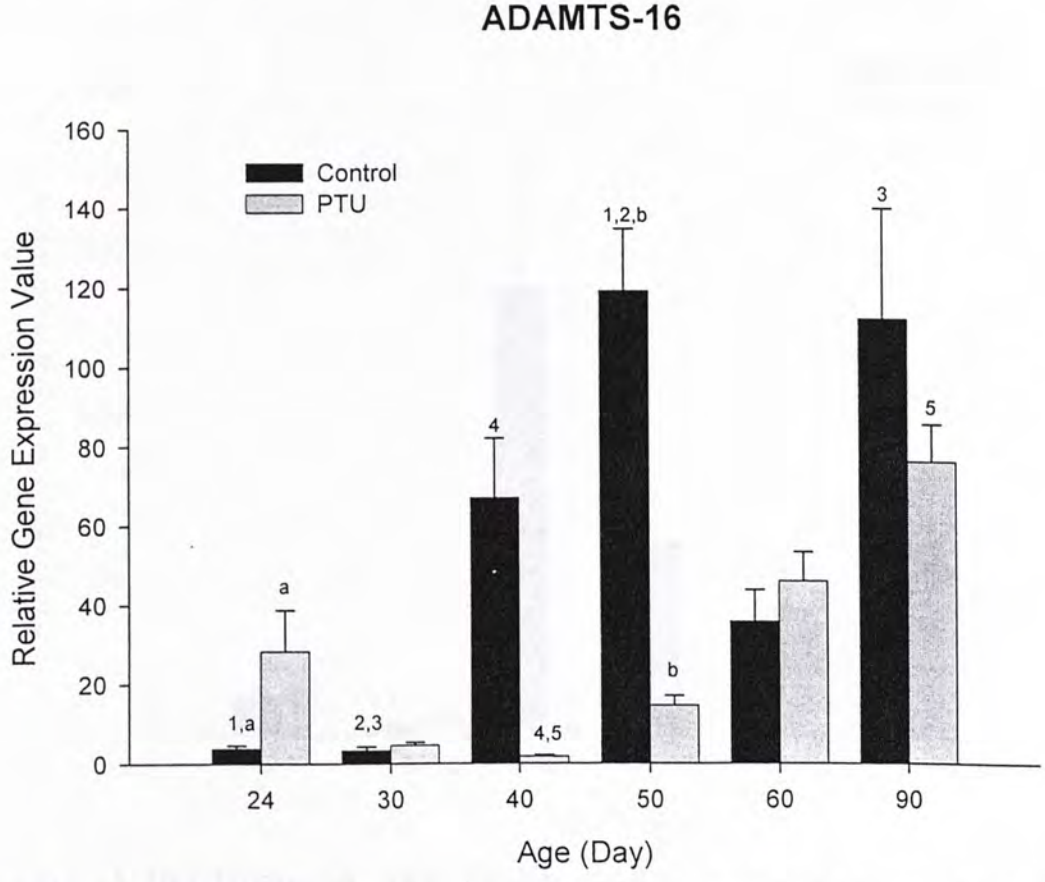


Fig 2.13 Developmental transcription profile of ADAMTS-16 gene. Numbers on top of columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Rank Sum test with $p<0.01$, b: Unpaired t-test with $p<0.001$.

MT3-MMP:

Expression of MT3-MMP (as shown in Fig 2.14) is similar with the other genes described before. Age, treatment and their interactions were significantly varied ($p<0.001$). Lowered expression is detected before day 30 when compared with that of the aged. Pair-wise comparison revealed significant down-regulation of gene suffered by hypothyroidism was appeared at day 40 to 50.

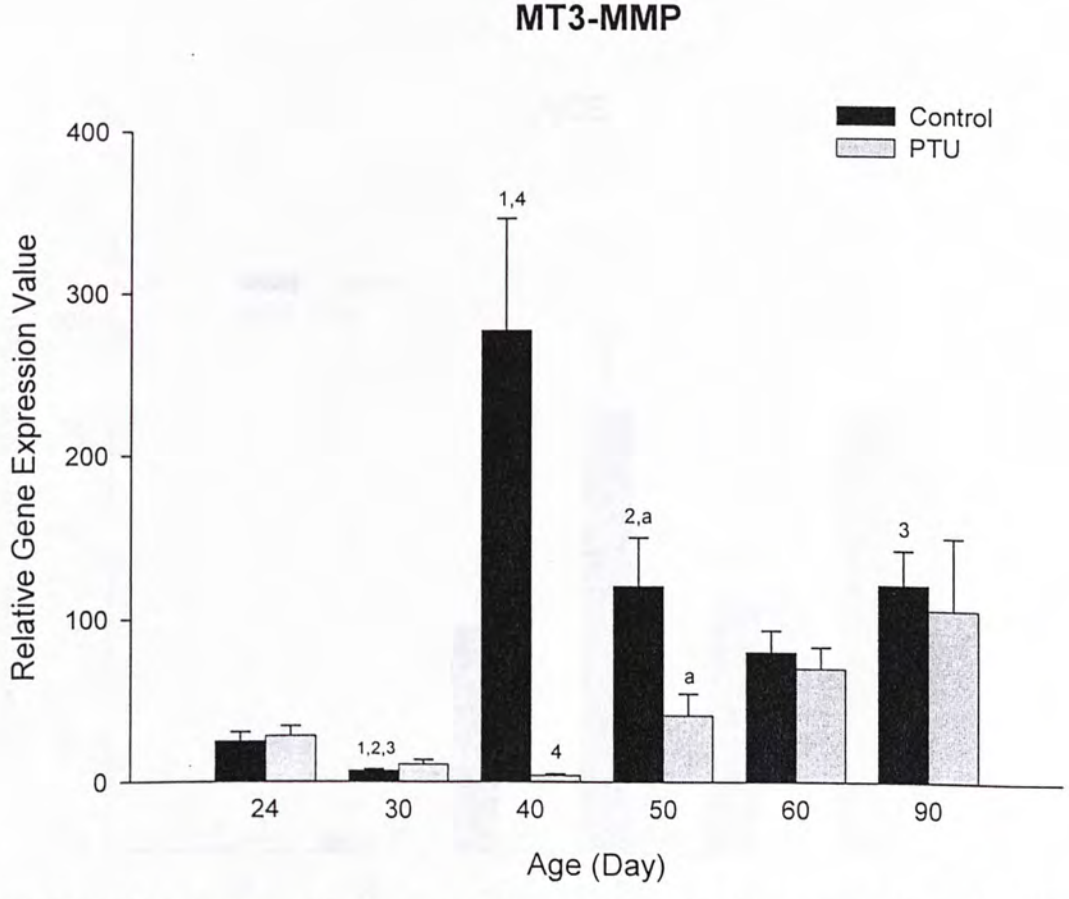


Fig 2.14 Developmental transcription profile of Membrane Type-3 Matrix Metalloprotease (MT3-MMP) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" show statistical significance by unpaired Student's t-test with $p<0.05$.

ACE:

As illustrated in Fig 2.15, the expression of ACE is similar with some of the ADAMs. Significant age, treatment and interactions were observed by 2-way ANOVA after $\log_{10}$ transformation ($p<0.001$). Expression of ACE before adult is extremely low, thus a significant higher transcription was illustrated when comparing between Day 90 verses that from Day 24 to 40 by Dunn's test. Pair-wise comparisons revealed distinct down-regulation for hypothyroid rats from age Day 30 to 50, while this returned to normal on Day 60.

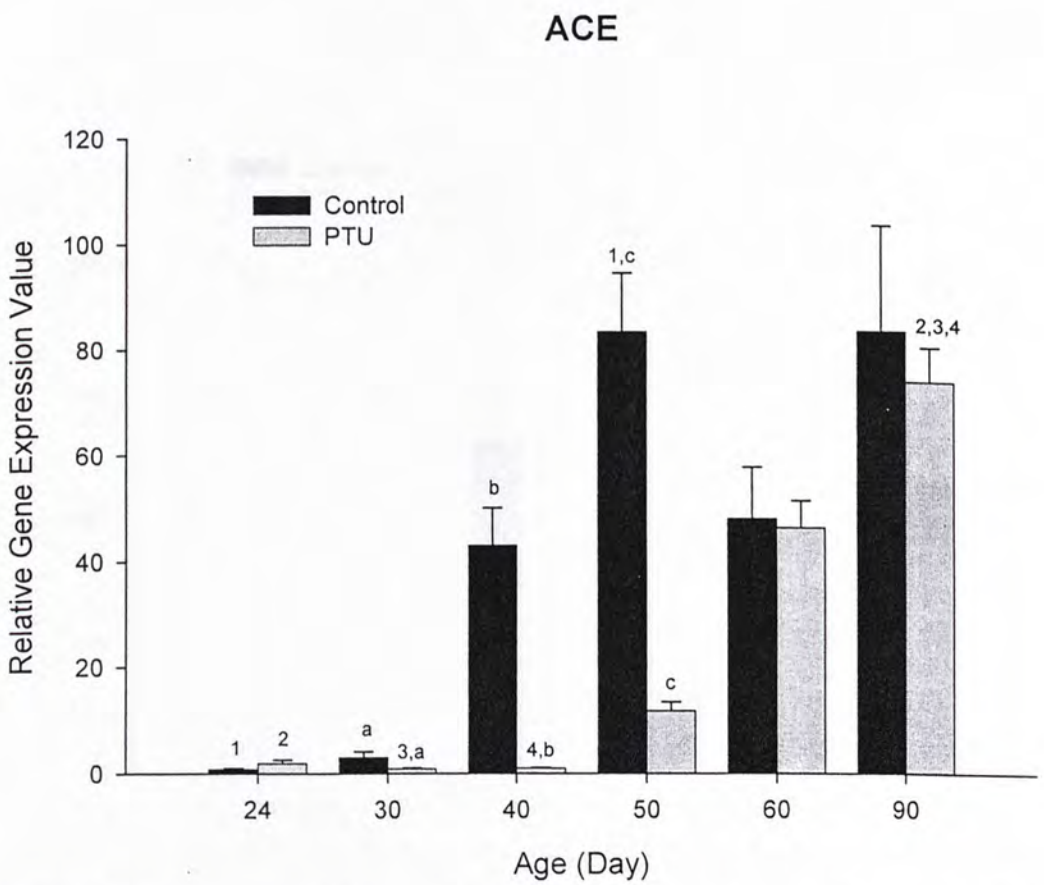


Fig 2.15 Developmental transcription profile of Angiotensin Converting Enzyme (ACE) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets a,b,c show statistical significance by Rank Sum test with $p<0.05$, <0.01 and <0.01 respectively.

Nep:

The expression profile of Nep, which is shown in Fig 2.16, is extremely similar with that of ACE. Age, treatment and their interactions were significantly affected by 2-way ANOVA after log10 transformation. Expression of Nep is also extremely low at early ages, thus drastic differences were found when comparing the expression at Day24 normal rats with that from Day 40 to 90. Hence distinct pair-wise down-regulations were observed in hypothyroid rats from Day 30 to 50.

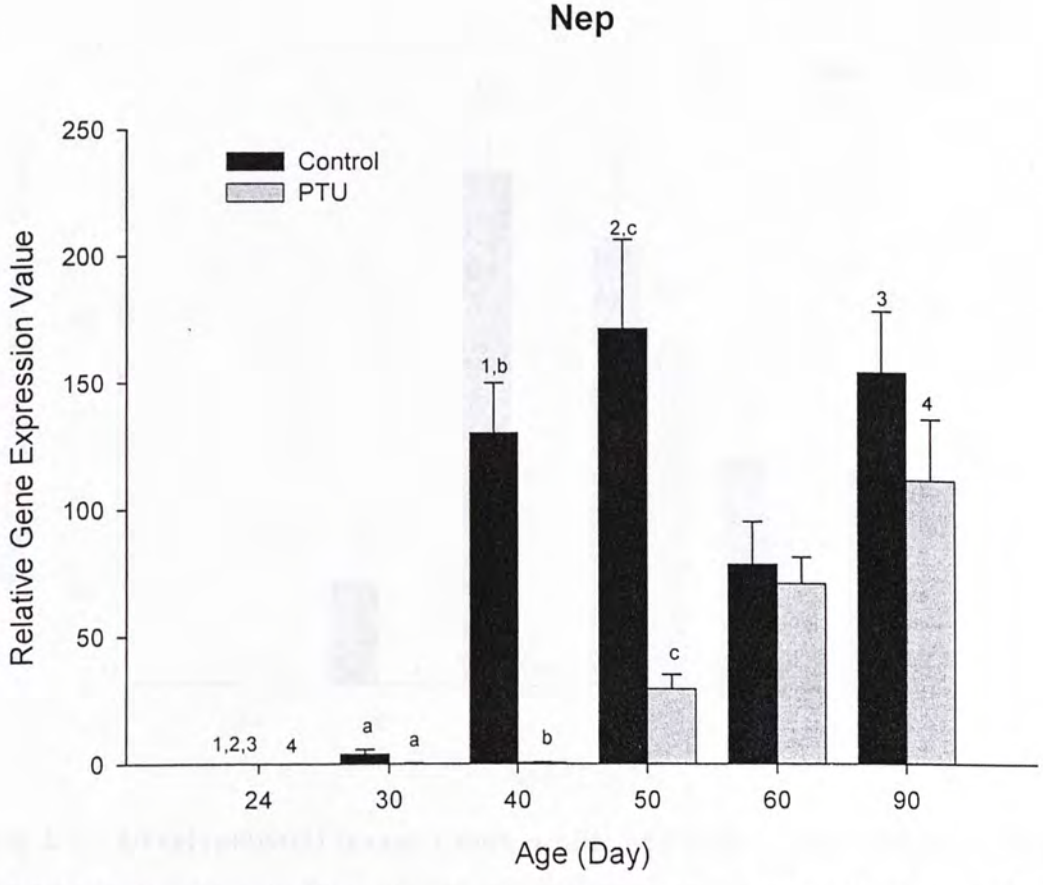


Fig 2.16 Developmental transcription profile of Neprilysin (Nep) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets a, b, c show statistical significance by Rank Sum test with $p<0.05$, <0.01 and <0.01 respectively.

PRSS21:

As shown in Fig 2.17, expression Profile of PRSS21 is similar with ADAM2, 3 and 5. Age, treatment and interactions were all significantly affected (Result of 2-way ANOVA with log10 transformation, $p<0.001$). Again, older rats have a significant higher expression of PRSS21 than young rats. Pair-wise comparisons revealed significant down-regulation of genes between Day 30 to 40, but also in Day 60.

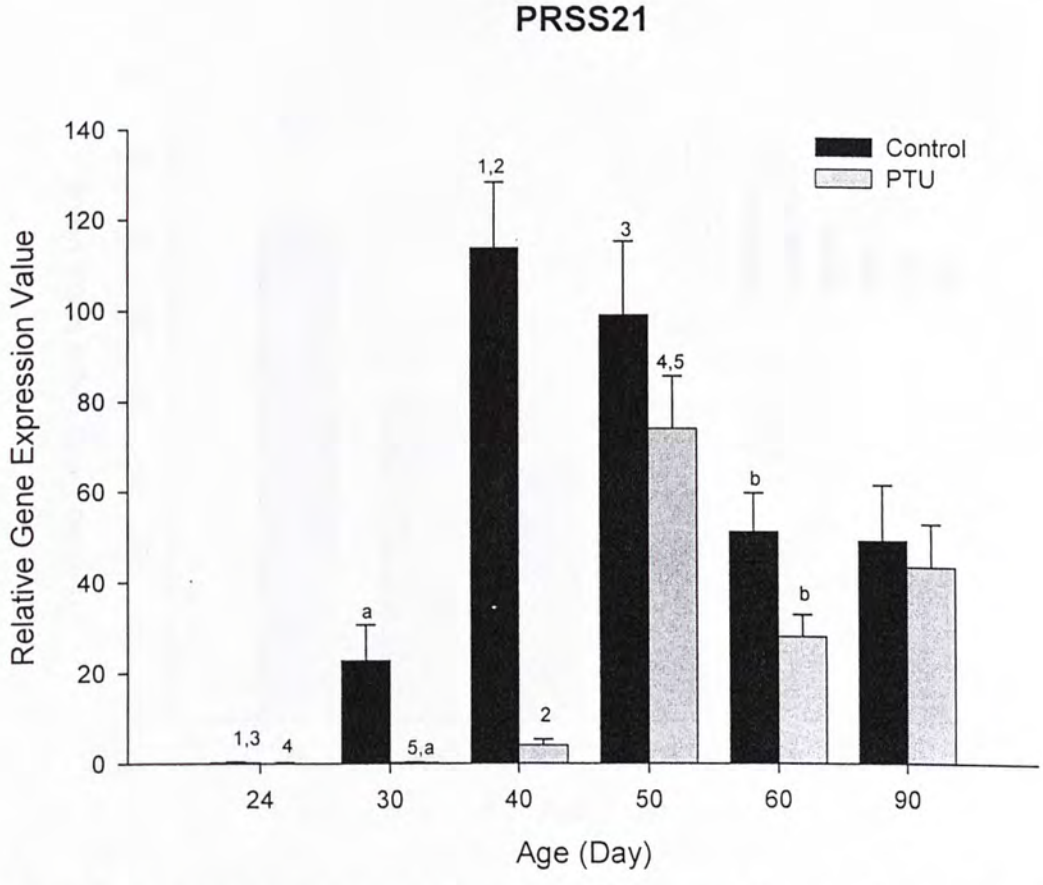


Fig 2.17 Developmental transcription profile of PRSS21 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Rank Sum test with $p<0.01$, b: Unpaired t-test with $p<0.05$).

tPA:

As illustrated in Fig 2.18, only a significant age effect ($p<0.001$) is found after Log_{10} transformation followed by 2-way ANOVA analysis. A gradual drop in tPA transcription was clearly seen. Dunn's rank sum test revealed a significant decrease from Day 24 vs Day 50, 60 and 90.

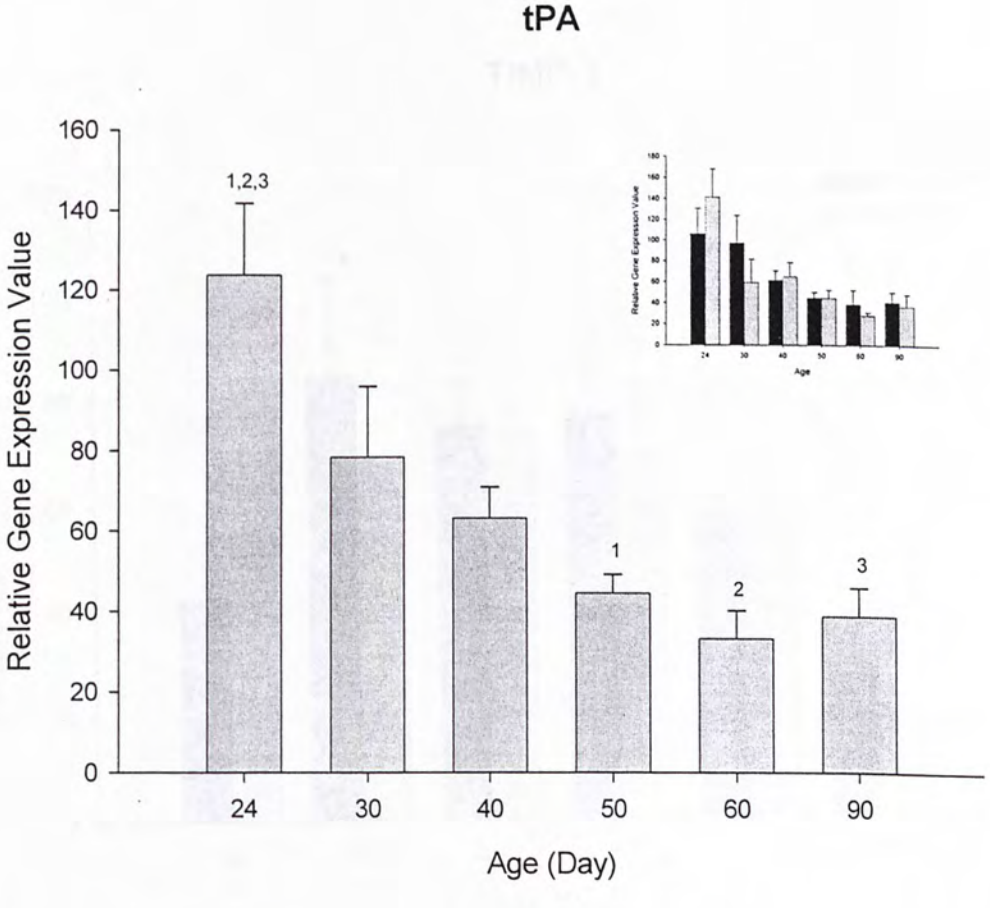


Fig 2.18 Developmental transcription profile of tissue Plasminogen Activator (tPA) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$.

TIMP-1:

Expression of TIMP-1 is age, treatment and interaction dependent (with $p<0.001$, <0.01 and <0.05 respectively) as shown in Fig 2.19. Transcription of both control and hypothyroid rats is quite constant, only a lowered expression at Day 90 was detected. Pair-wise Students' t-test only showed a significant down-regulation at Day 30 in PTU-treated rats.

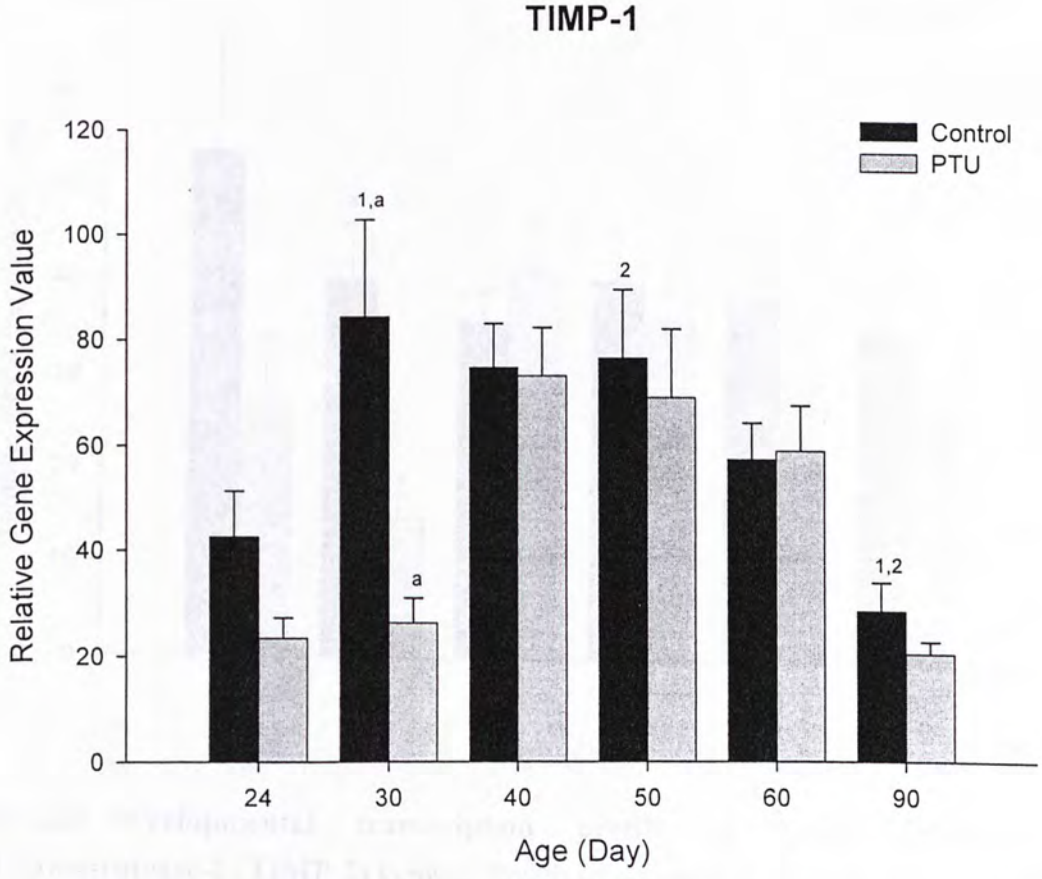


Fig 2.19 Developmental transcription profile of Tissue Inhibitor of Metalloprotease-1 (TIMP-1) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" shows statistical significance by Unpaired Student's t-test with $p<0.01$.

TIMP-2:

Expression of TIMP-2 only showed a moderate age dependency ($p<0.01$), which is shown in Fig 2.20. No treatment or interaction variation can be observed. But a significant down-regulation of TIMP-2 is shown under hypothyroidism at Day 30.

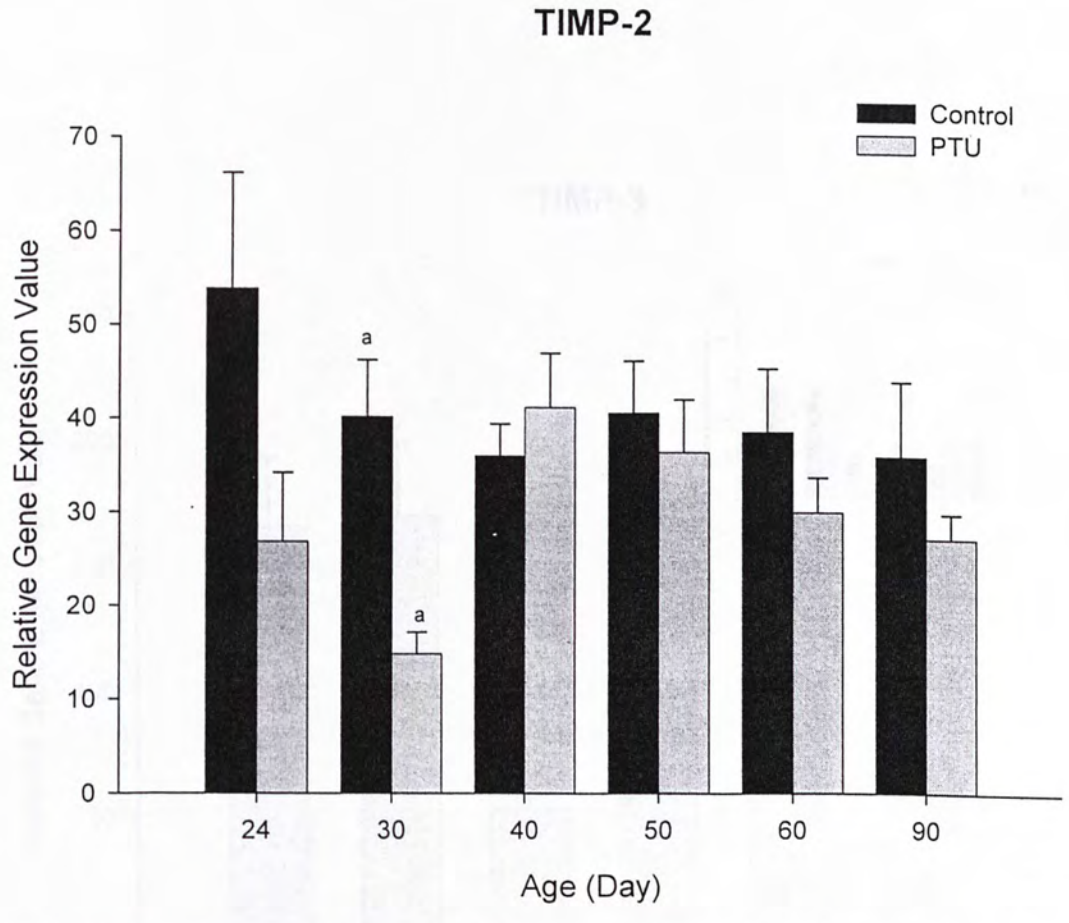


Fig 2.20 Developmental transcription profile of Tissue Inhibitor of Metalloprotease-2 (TIMP-2) gene. a: Result of unpaired Student's t-test with $p<0.01$.

TIMP-3:

As shown in Fig 2.21, TIMP-3 only showed an age-dependent effect after Log_{10} transformation ($p<0.001$). Dunn's multiple comparison showed a significantly lowered transcription at Day 40, when compared with that of early ages, suggesting a tread of age-dependent down-regulation. No significant pair-wise differences when comparing the expression between normal and hypothyroid rats at any ages.

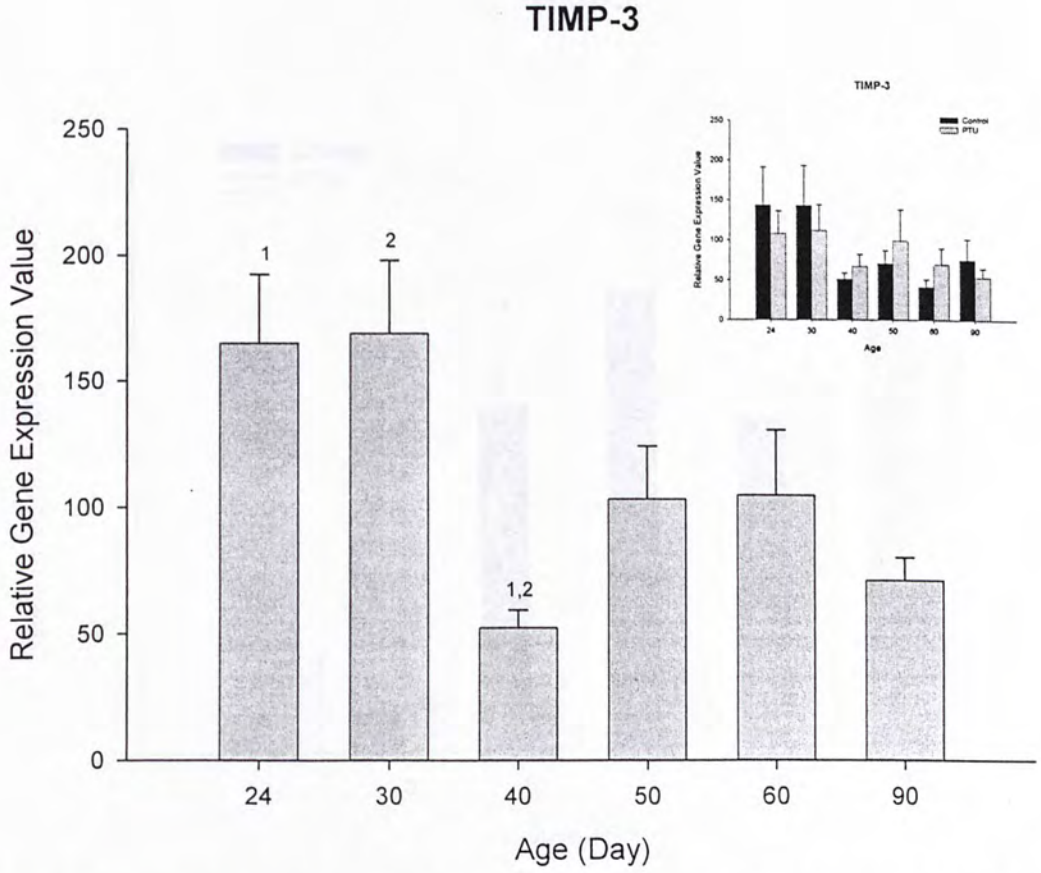


Fig 2.21 Developmental transcription profile of Tissue Inhibitor of Metalloprotease-3 (TIMP-3) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$.

TIMP-4:

As illustrated in Fig 2.22, TIMP-4 was the only TIMP family member that showed the characteristic expression profile like the pervious mentioned candidates. After square-root transformation, transcription of TIMP-4 is highly age, treatment or interaction dependent ($p<0.001$). Expressions at early ages (either in control or hypothyroid rats) are lowered at early ages. Pair-wise comparisons revealed significant drop in expression in TIMP-4 between Day 40 to 50 when pups suffered hypothyroidism

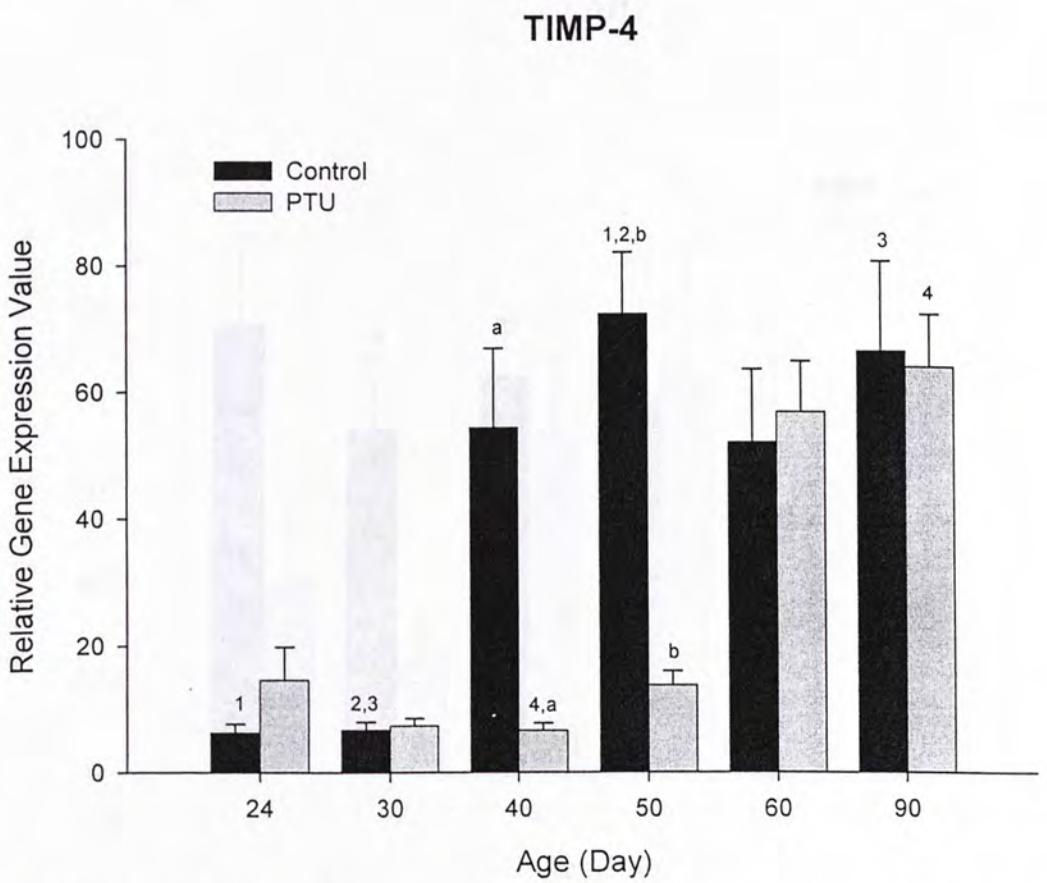


Fig 2.22 Developmental transcription profile of Tissue Inhibitor of Metalloprotease-4 (TIMP-4) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons, a: Unpaired t-test with $p<0.01$, b: Rank-Sum test with $p<0.001$.

α2MG:

As illustrated in Fig 2.23, effects of age, treatment and their interactions significantly affected the transcription of α2MG gene (results of 2-way ANOVA $p<0.01$). Multiple comparisons with Dunn's test revealed no significant differences between any interesting data pairs. Only a significant higher transcription of this gene is found at day 30.

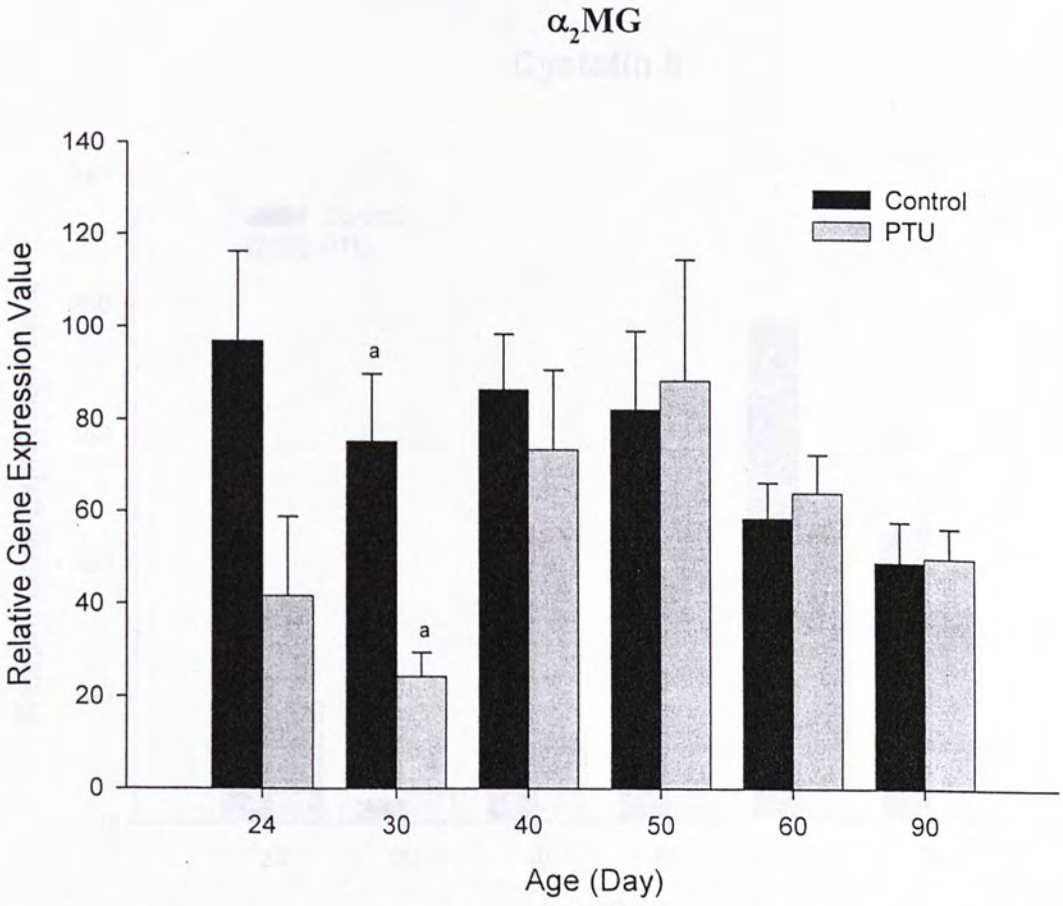


Fig 2.23 Developmental transcription profile of alpha-2 macroglobulin (α_2MG) gene. a: Result of unpaired Student's t-test: with $p<0.01$.

Cystatin 8:

As shown in Fig 2.24, significant age, treatment and interaction effect was illustrated after Log_{10} transformation followed by 2-way AVOVA with all p-value lower than 0.001. A graduate increase in transcription is observed, where expression of Cystatin 8 at Day 30 is significantly lower than that of the aged day 40 to 90 rats by Dunn's test. Pair comparison also revealed significant down-regulation of hypothyroid rats at Day 40 and 50

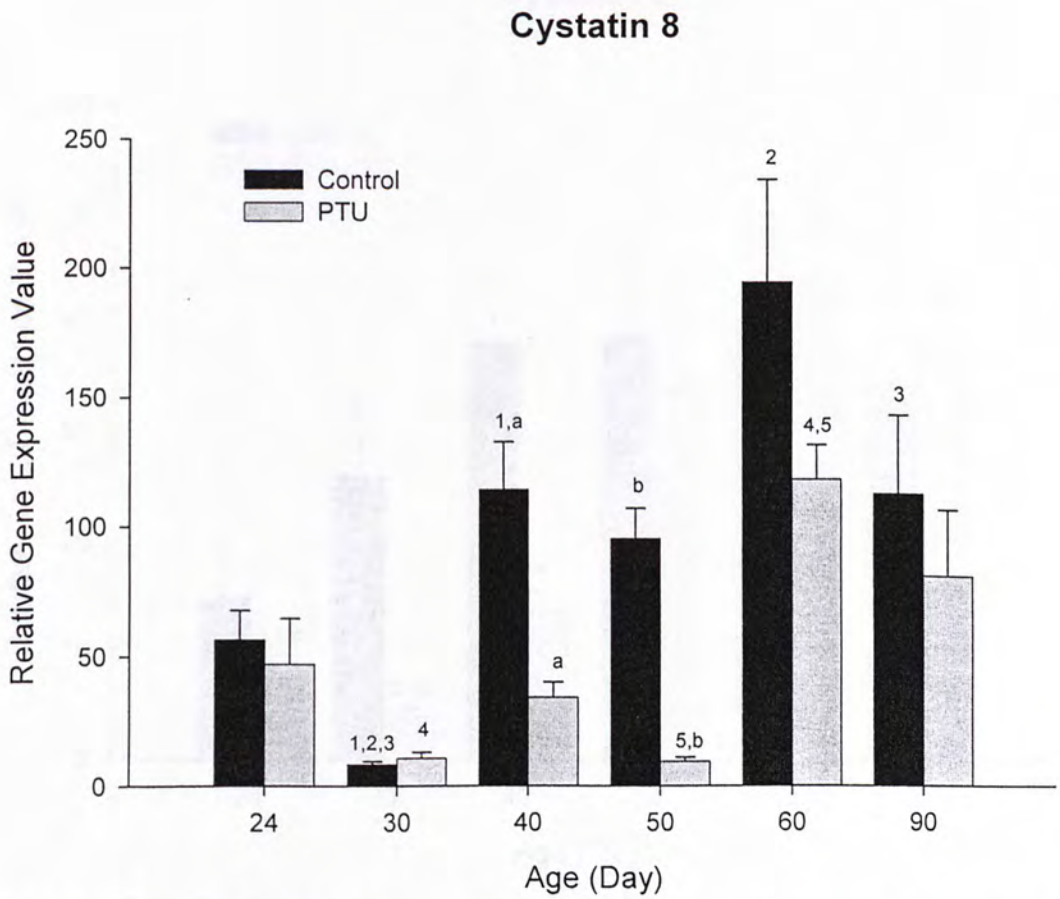


Fig 2.24 Developmental transcription profile of Cystatin 8 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Rank Sum test with $p<0.01$, b: Unpaired Student's t-test with $p<0.01$.

Cystatin C:

As illustrated in Fig 2.25, expression profile of Cystatin C is different with that of its family Cystatin 8. Again, expression of Cystatin C is dependent on age, treatment and its interaction after $\log_{10}$ transformation with $p<0.001$. Dunn's test showed significant up-regulation of expression at Day 60 where compared with that from early Day 24 to Day 40 in PTU-treated rats. Pair-wise comparisons also revealed a considerable down-regulation of Cystatin C expression from Day 24 to 40 in the neonatal hypothyroid rats.

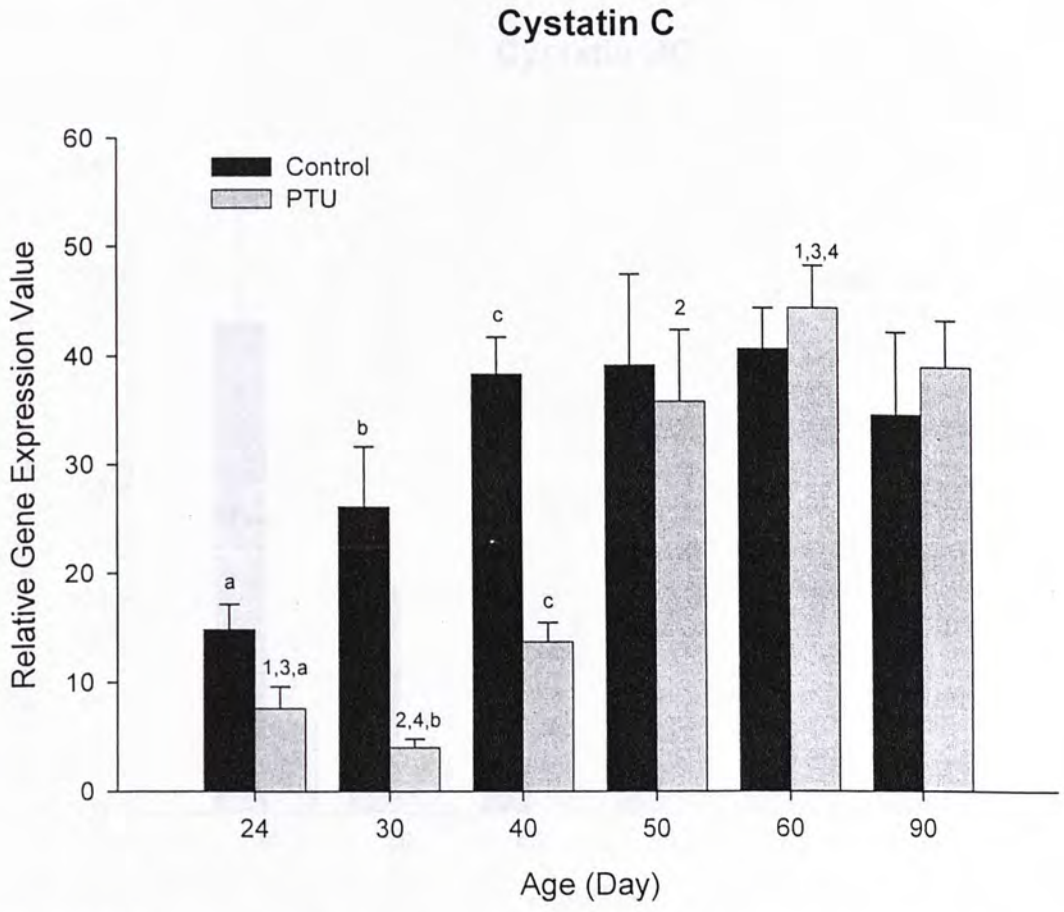


Fig 2.25 Developmental transcription profile of Cystatin C gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a, c: Unpaired Student's t-test with $p<0.05$ and <0.001 respectively. b: Non-parametric Rank-Sum test with $p<0.01$.

Cystatin SC:

Fig 2.26 shows the expression profile of Cystatin SC. Significant age, treatment and interaction dependencies were illustrated in the expression, while age effect is more prominent one ($p<0.001$; $p<0.05$ in treatment effect). An age-dependent down-regulation can be clearly seen, where the transcription is significantly lowered in Day 90. Considerable down-regulation was suffered in hypothyroid rats between Day 24 to Day 30.

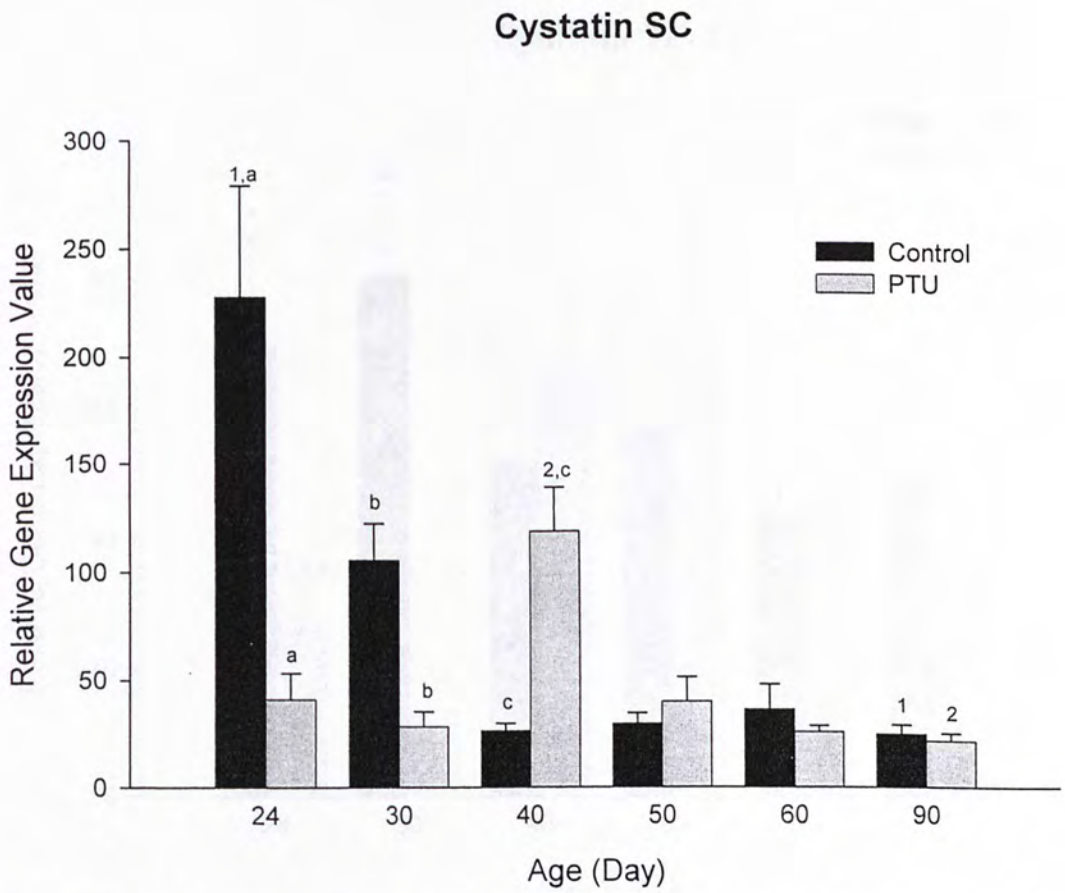


Fig 2.26 Developmental transcription profile of Cystatin SC gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a, b: Unpaired t-test with both $p<0.01$, c: Rank-Sum test with $p<0.05$.

Cystatin TE-1:

As shown in Fig 2.27, the expression of Cystatin TE-1 is distinctive to the other cystatins examined in this study. Significant treatment and interaction effects were illustrated ($p<0.001$), while a moderate age-dependent effect is also found ($p<0.05$). Transcription of this gene in control rats is more or less constant, but lowered expression is initiated in rats suffered from hypothyroidism at pre-pubertal ages. Pair comparisons showed a significant down-regulation at Day 24 and 30 in the PTU-treated rats.

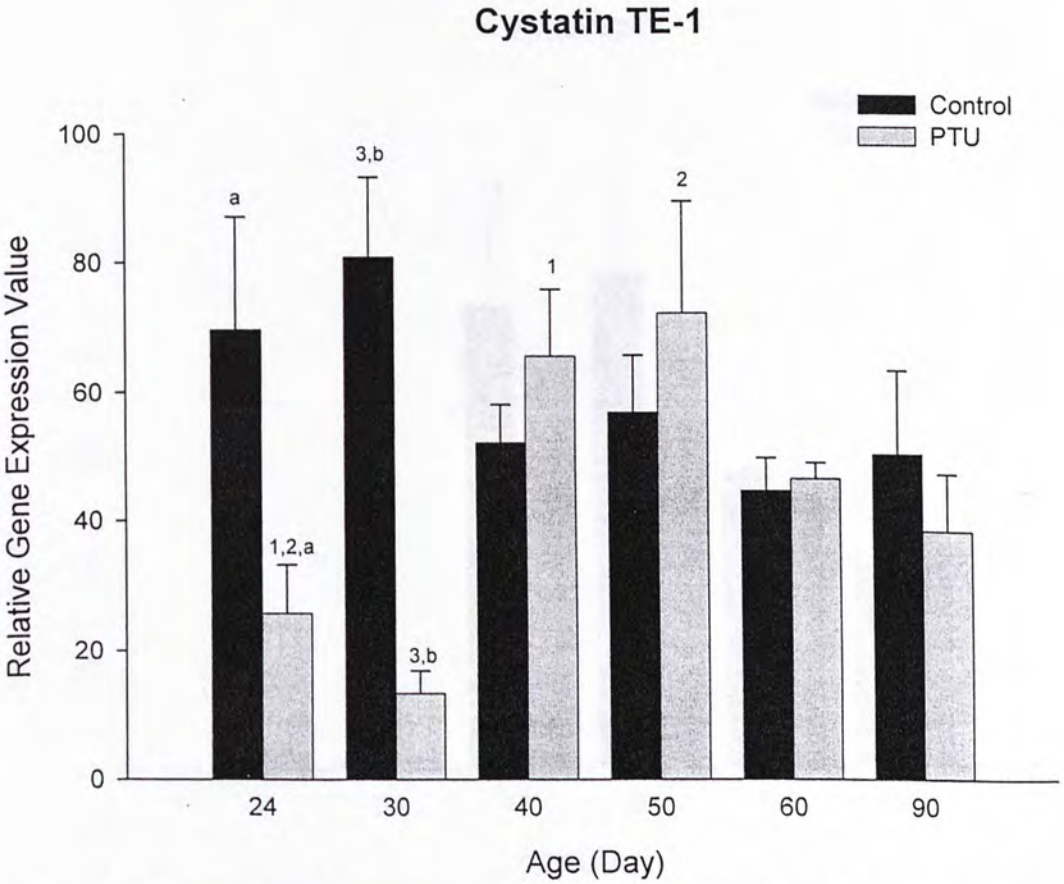


Fig 2.27 Developmental transcription profile of Cystatin TE-1 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a: Rank Sum test with $p<0.05$, b: Unpaired Student's t-test with $p<0.05$.

Bikunin:

The transcription of Bikunin, which is illustrated in Fig 2.28, is very similar to those of ADAM4 and ADAM6. Results of two-way ANOVA showed significant age, treatment and their interaction variations on their transcription with $p<0.001$ after $\log_{10}$ transformation. Older rats seem to have a higher transcription levels than that of younger rats. Expression is extremely low before Day 30, but a rapid boost in transcription was seen at Day 40 and maintained at peak afterwards. Significant down-regulation of Bikunin was observed in hypothyroid pups between Day 40 and Day 50, where transcription restored to normal at Day 60.

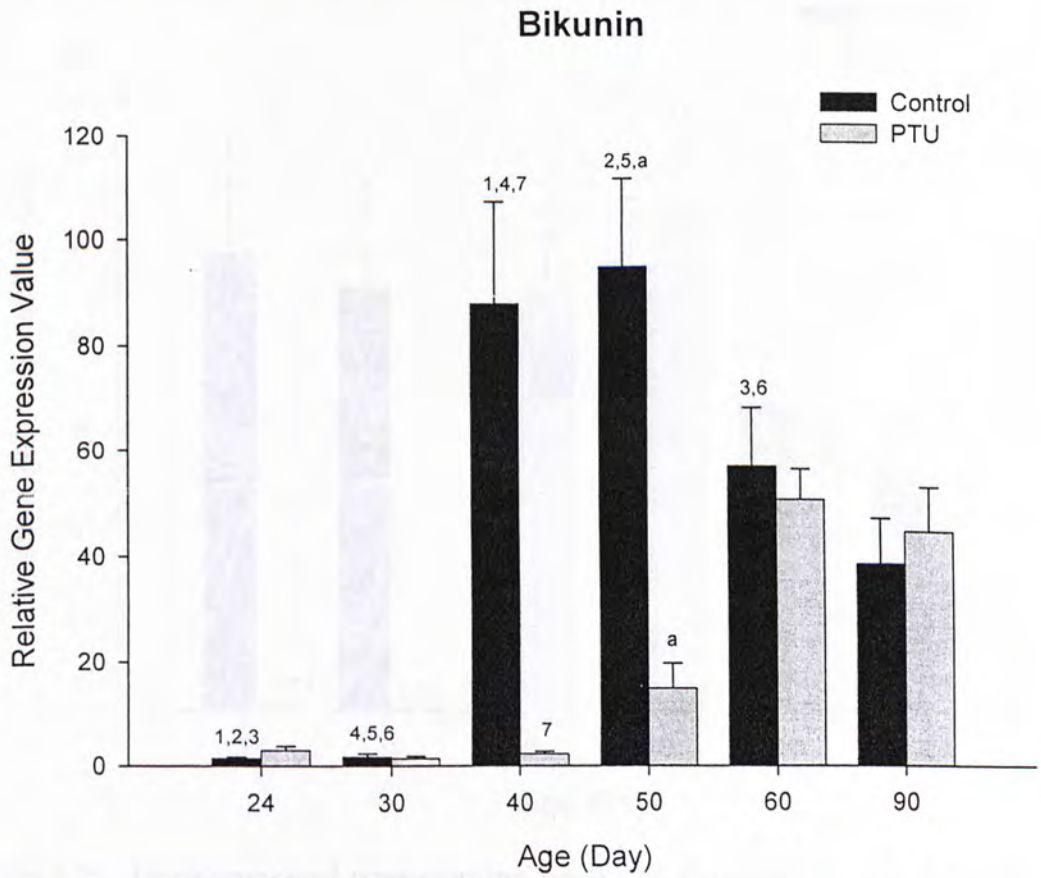


Fig 2.28 Developmental transcription profile of Bikunin gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" show statistical significance by Unpaired Student's t-test with $p<0.01$.

PCI:

Expression profile of PCI is unique within this study as shown in Fig 2.29. Age, treatment and interactions were significantly affected ($p<0.001$). Transcriptions of PCI in the neonatal hypothyroid rats are extremely low at Day 24 to Day 30. Thus significant elevated expression can be seen from Day 24 to day 30 verses that of Day 40 to Day 50. Drastic down-regulation can be seen in hypothyroid rats from Day 24 to 30 when compared with normal, but expression recovered as soon as at Day 40.

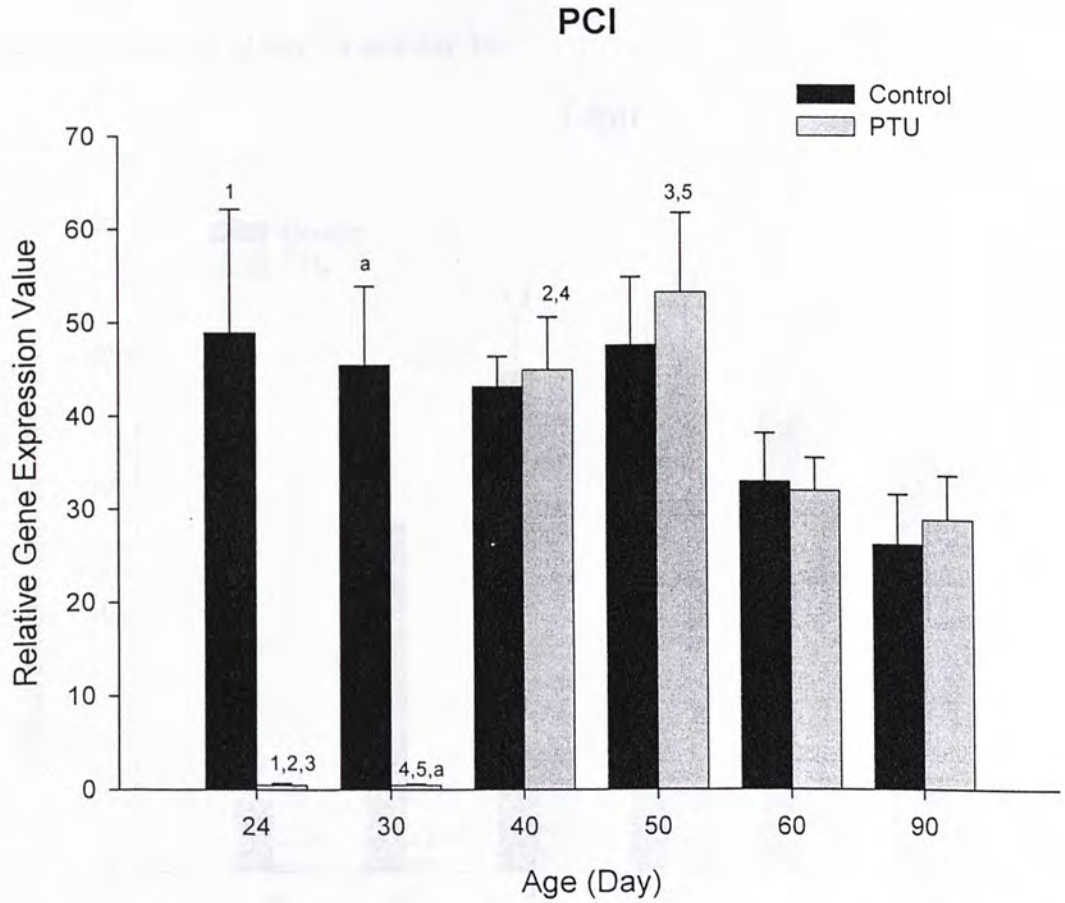


Fig 2.29 Developmental transcription profile of Protein C Inhibitor (PCI) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" shows statistical significance by Rank-Sum test with $p<0.01$.

Clgn:

Very similar to the ADAM 2 gene, transcription of Clgn gene is high significantly affected by age, treatment and their interaction from 2-way ANOVA after square root transformation with $p<0.001$ (Fig 2.30). Multiple comparisons with Dunn's test reveal significantly higher transcription of Clgn in day 40 control rat than those at day 24. Between the neonatal hypothyroid rats at day 24 and day 50, 60 and those between day 30 and 50, 60, significantly higher transcription of this gene is indicated in the older rats. At day 40, significantly higher transcription of Clgn is still indicated in the control. Using pair comparison, significantly higher transcription level is observed at day 24 and day 30.

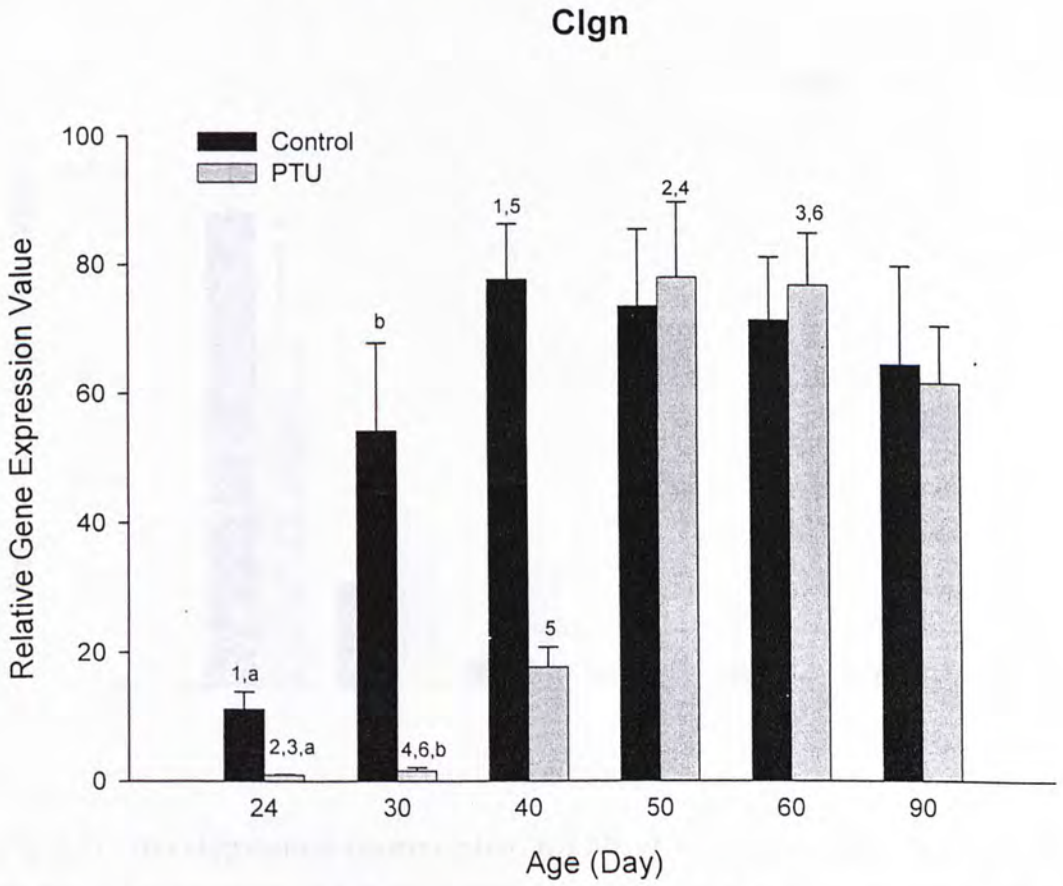


Fig 2.30 Developmental transcription profile of Calmegrin (Clgn) gene. a: Unpaired Student's t-test with $p<0.01$, b: Mann-Whitney's Rank Sum test with $p<0.05$, number pairs revealed significant differences by Dunn's multiple comparison with all $p<0.05$.

Claudin-1:

A significant age-dependent down-regulation is clearly illustrated in Fig 2.31 with $p<0.001$. Moderate treatment and interaction effect are also observed with $p<0.05$ and <0.01 respectively. High expression during early ages revealed significant difference when compared with that at day 60 to 90 by multiple comparisons. Pair-wise comparisons revealed significant differences between normal and hypothyroid groups at day 40 and 50.

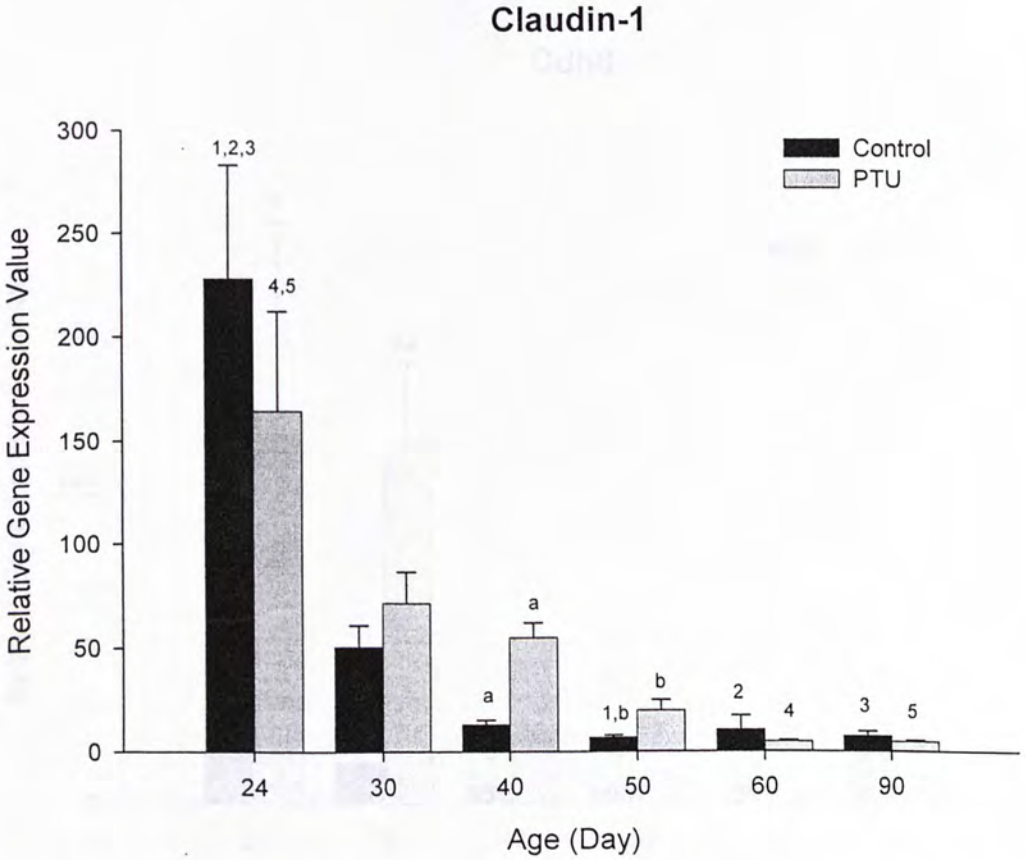


Fig 2.31 Developmental transcription profile of Claudin-1 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparisons. a,b: Significant difference revealed by unpaired Student's t-test with $p<0.001$ and $p<0.05$ respectively.

Cdh-8:

As illustrated in Fig 2.32, transcription of Cdh-8 is entirely unique from other genes being exanimate. Significant age, treatment and interaction effect was seen ($p<0.001$) by two-way ANOVA after Log_{10} transformation, but the effect of treatment is less prominent ($p<0.01$). Both control and PTU-treated rats possess a drastic down-regulation when the rat grows up. Unpaired Students' t-test revealed significant up-regulation in Cdh8 transcription under neonatal hypothyroidism before their adulthood. A moderate increased expression in control rats was also found on Day 90.

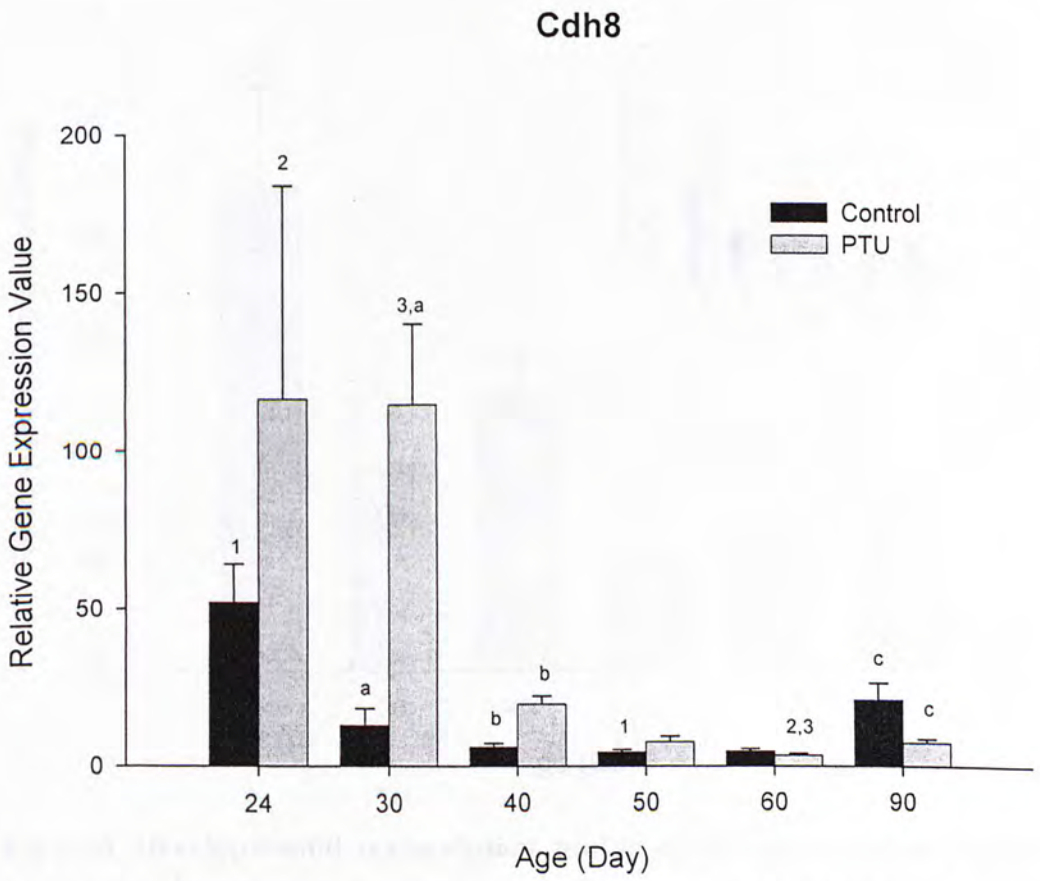


Fig 2.32 Developmental transcription profile of Cadherin-8 (Cdh-8) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets show statistical significance by pair comparison, a, b, c: unpaired Student's t-test with $p<0.01$, <0.001 and <0.05 respectively.

Syndecan-4:

As shown in Fig 2.33, the expression profile of syndecan-4 is similar with that of tPA. Only a significant age-dependent down-regulation can be observed ($p<0.001$). Dunn's multiple comparisons revealed significant reduction of syndecan-4 expression from day 50 to day 90 when contrasted with that of the high transcription at day 24.

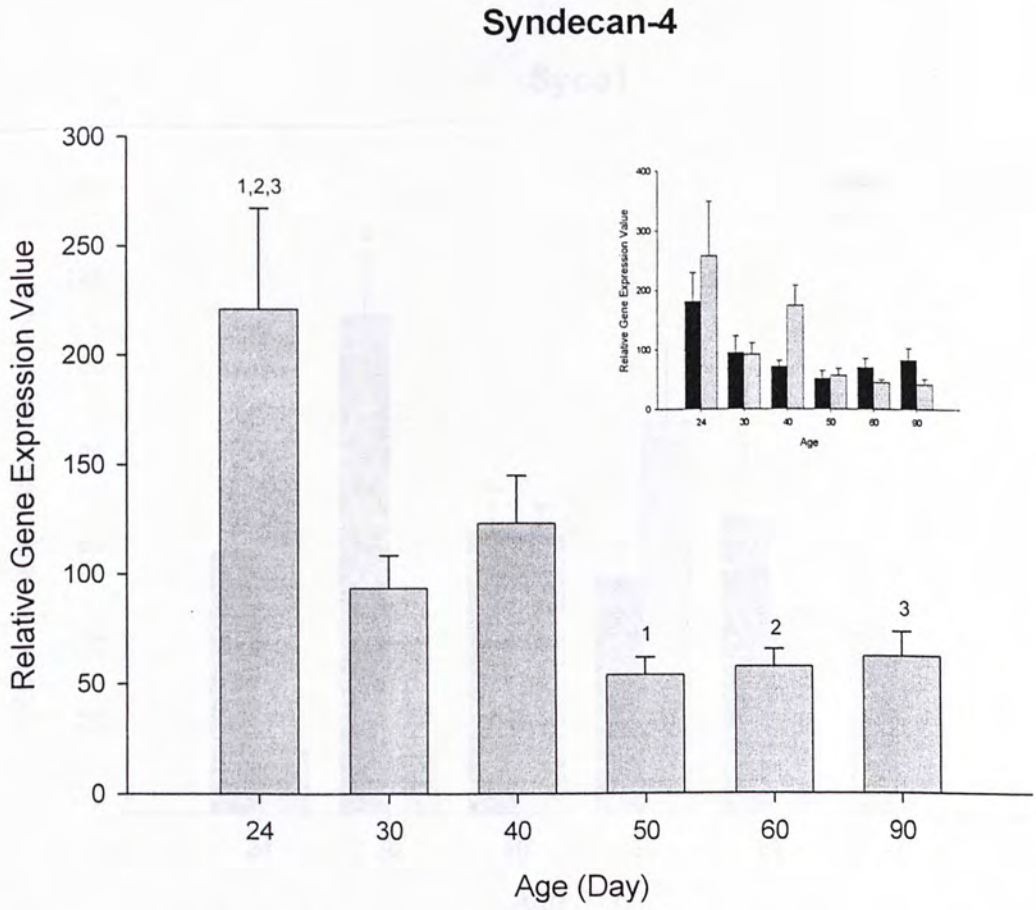


Fig 2.33 Developmental transcription profile of Syndecan-4 gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$.

Sycp1:

Significant age, treatment and interaction variations were illustrated in Fig 2.34 by two-way ANOVA after square-root transformation. (Treatment and interaction effect contributes with $p<0.001$, while age effect was $p<0.01$). Expression of Sycp1 in control remains more or less constant, but significantly lowered expressions were found from day 24 to 30 when compared with the plateau. Pair-wise comparisons revealed moderate depression in gene transcription from Day 24 to Day 30.

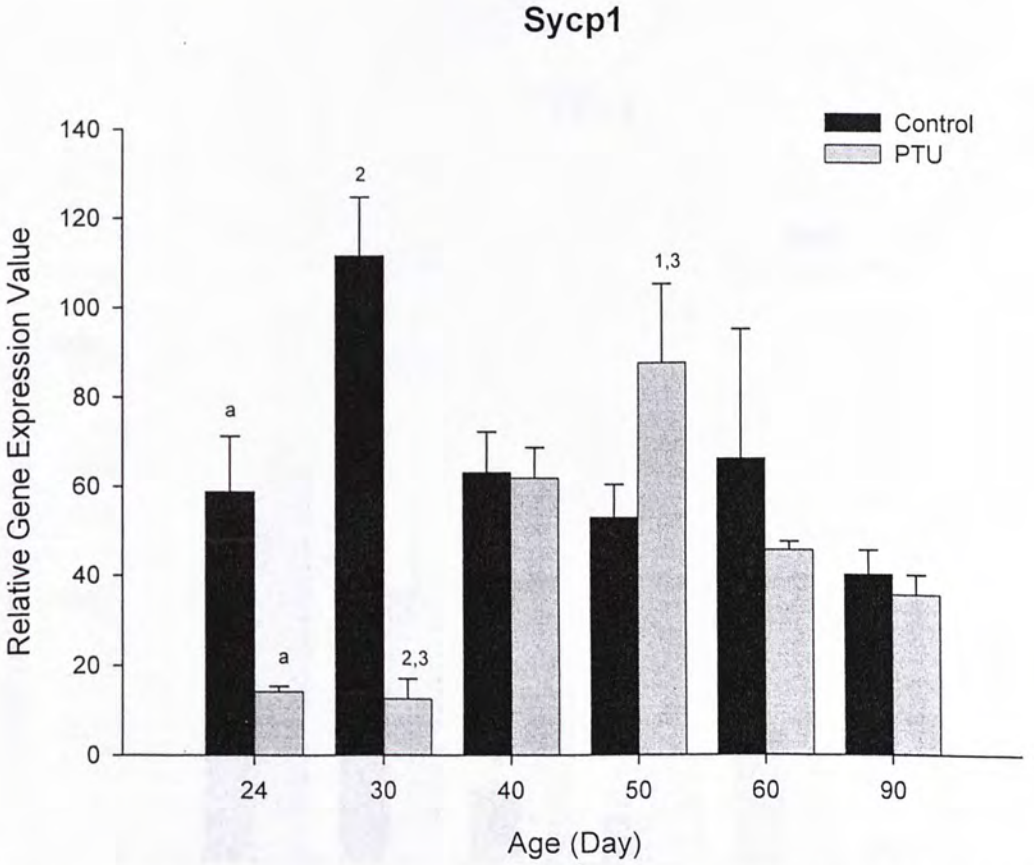


Fig 2.34 Developmental transcription profile of Synaptonemal Complex Protein 1 (Sycp1) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" show statistical significance by unpaired Student's t-test with $p<0.01$.

TRα1:

Transcription of TRα1 gene is significantly affected by both age and treatment ($p<0.001$; results of 2-way ANOVA after Log_{10} transformation) but not their interaction. Multiple comparisons after ANOVA by rank using Dunn's test indicate significant higher levels of transcription of TRα1 in the neonatal hypothyroid rat testis at day 24, 30, 40 than at day 90. Due to the large variations of the transcription of this gene at day 24 and 30, only a significant higher transcription of thyroid hormone receptor is found at day 40.

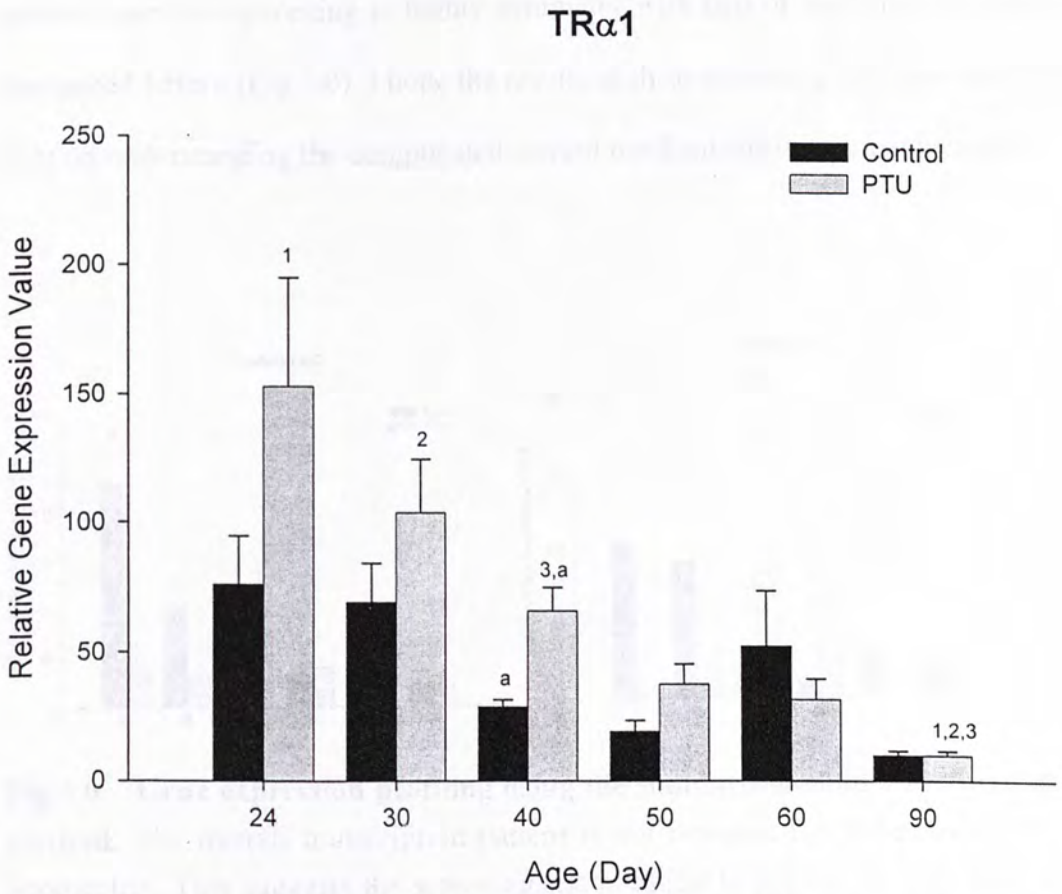


Fig 2.35 Developmental transcription profile of Thyroid Hormone Receptor α1 subunit (TRα1) gene. Numbers on top of the columns show statistical significance between the data pairs by Dunn's test with $p<0.05$. Paired alphabets "a" show statistical significance by unpaired Student's t-test with $p<0.01$.

Screening Data

In order to facilitate the study of transcription profiling in an economical way, a screening method was adopted to reduce the sample numbers in PCRs with certain representations to the final pictures of the profiling. Sample cDNAs collected within same age and treatment group were pooled together to make up a library. Twelve libraries made of six age groups (at day 24, 30, 40, 50 60 and 90) and two treatment groups (Control and PTU-induced hypothyroidism) were subjected to Real-Time PCR with replicates as previously described. This approach of using the screening idea can retain the accuracy and representation of the transcription profiling, since the overall pattern from this screening is highly symmetry with that of the standard method as mentioned before (Fig 3.0). I hope the results of these screening tests can shed certain light on understanding the complicated control mechanisms in spermatogenesis.

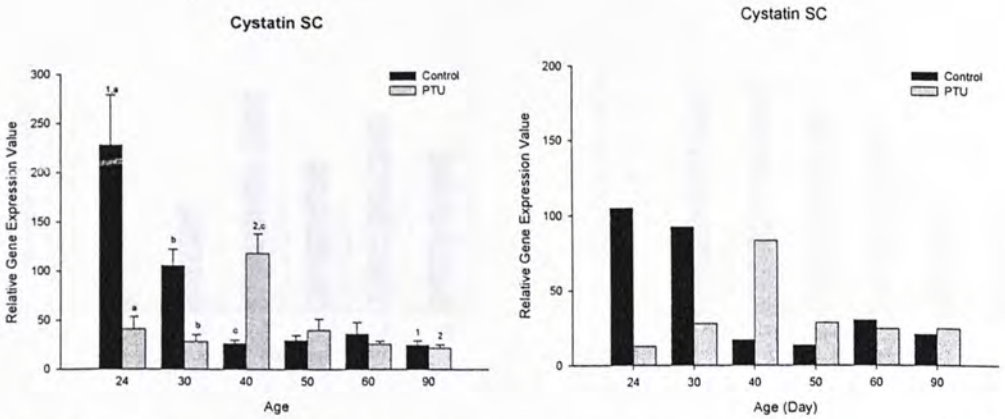


Fig 3.0 Gene expression profiling using the standard method and the screening method. The overall transcription pattern is not deviated much between these two approaches. This suggests the screening test is useful to provide an over-view on the transcription of a target gene without the possibility of statistical analysis.

Transcriptional Modulators:

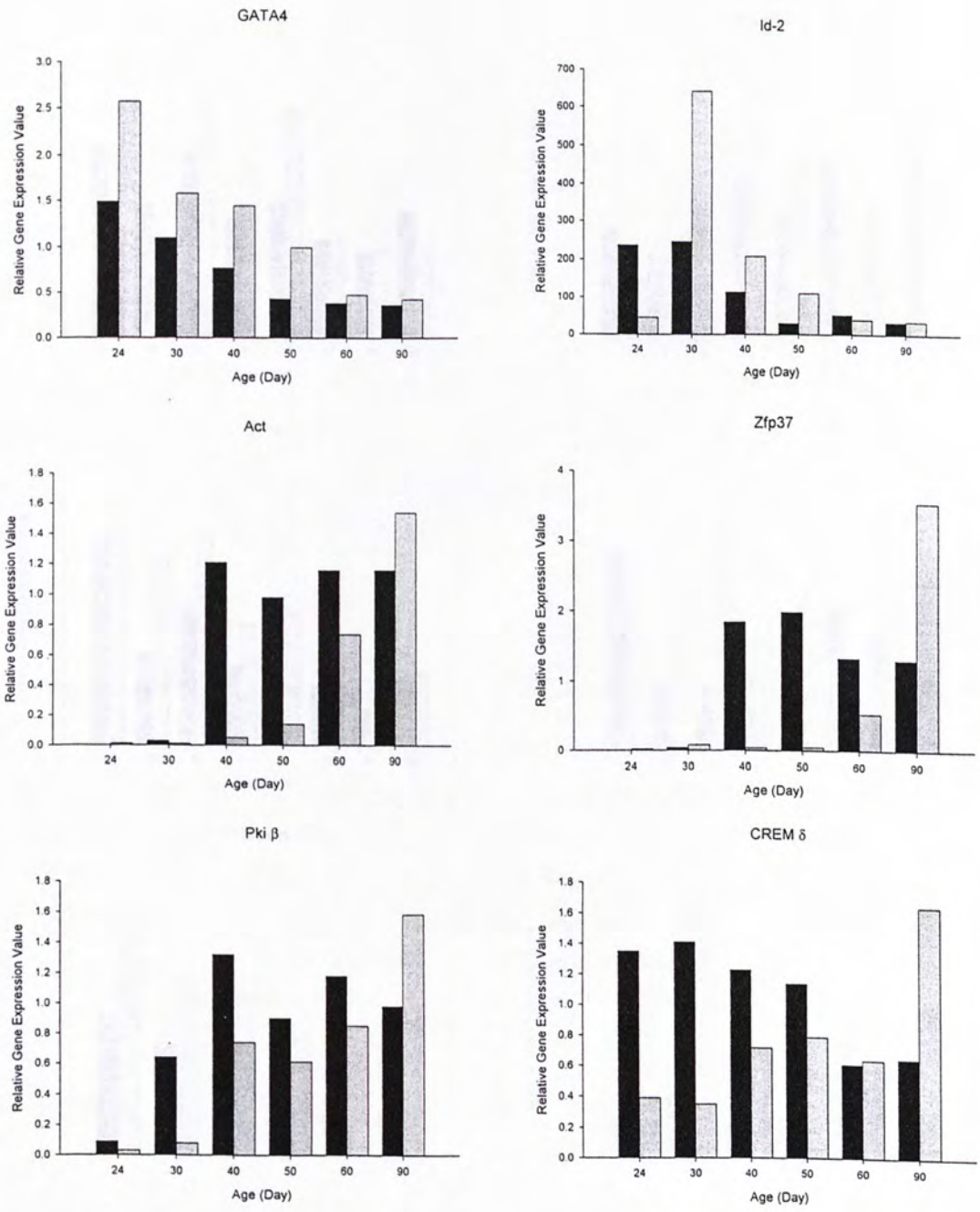


Fig 3.1 Screening transcription profiles of some transcription modulators. GATA4 and Id-2 are mainly expressed in spermatogonia. Act and Zfp37 are predominantly expressed in post-meiotic spermatids. Pki β is "meiotic", while the expression of CREMδ seems to have a pattern of the mixture of somatic and meiotic profile.

Signal Transduction:

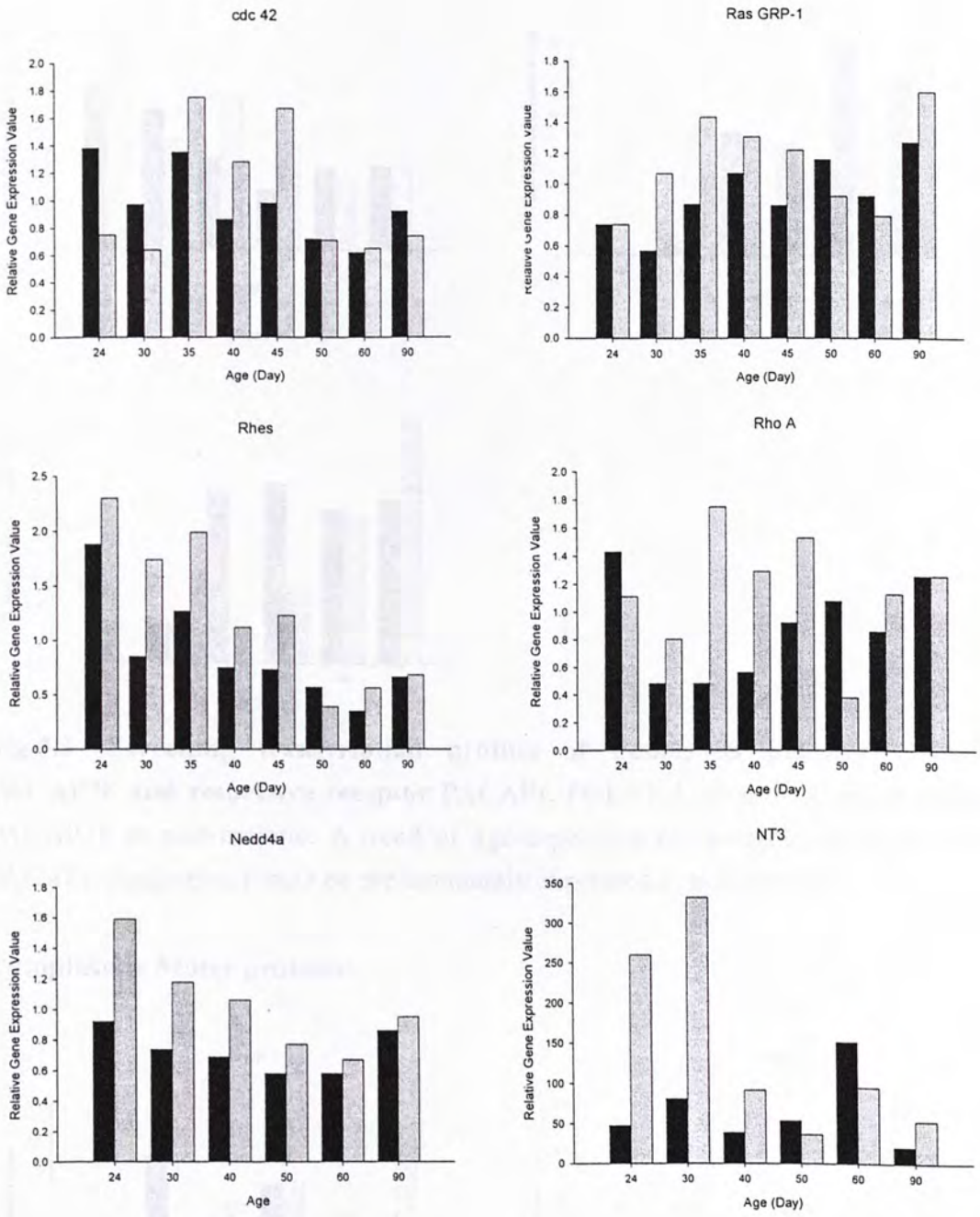


Fig 3.2 Screening transcription profiles of some signal transducers and messengers. Expressions of small GTPase like Rho subfamily of cdc42 and RhoA as well as Ras GRP-1 are constant throughout age, except Rhes which have an age-dependent down-regulation trend. Nedd4a is classified in the "Somatic" cluster where its main site of expression is in CNS, but abundant amount is present in other tissues. NT3 has a unique profile similar with that of TRα1 and Cdh-8, elevated transcription was identified in early ages under neonatal hypothyroidism.

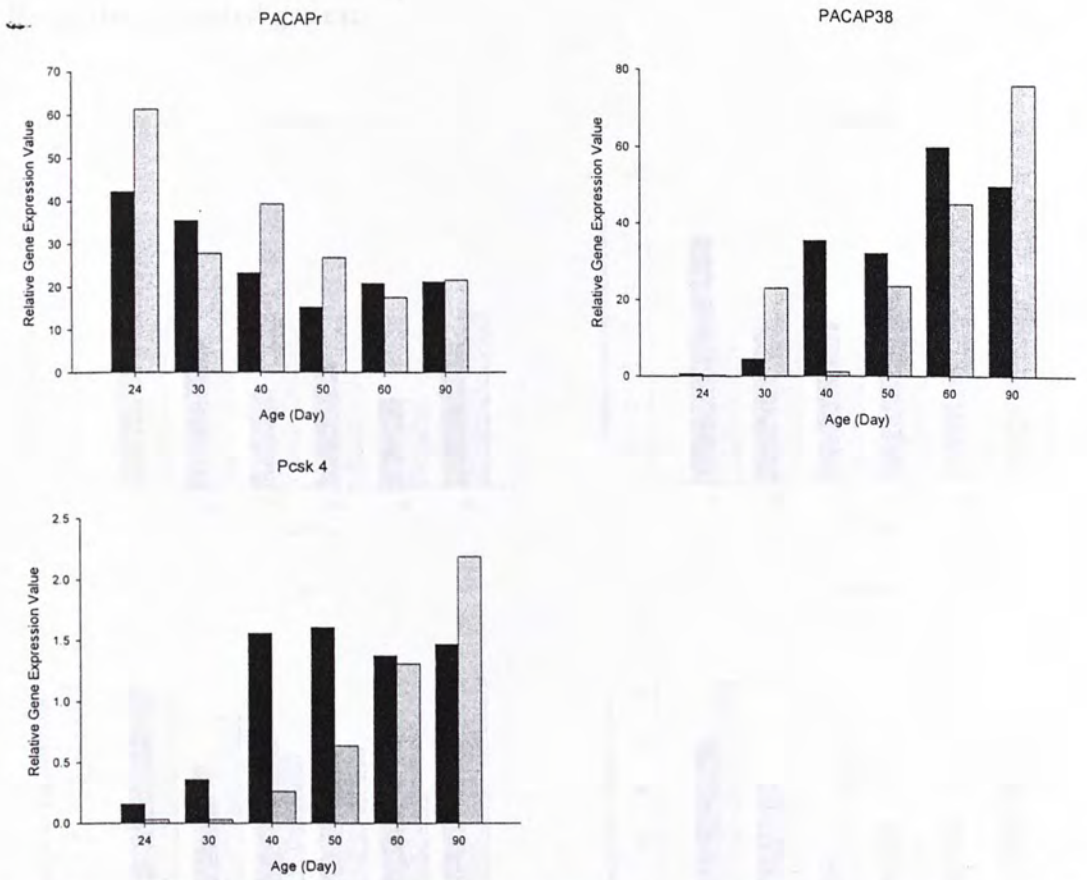


Fig 3.3 Screening transcription profiles of Pcsk4, its putative substrate PACAP38 and respective receptor PACAPr. Pcsk4 is known to be meiotic, while PACAP38 is post-meiotic. A trend of age-dependent down-regulation is shown in PACAPr, suggesting it may be predominantly expressed in Sertoli cells.

Cytoplasmic Motor proteins:

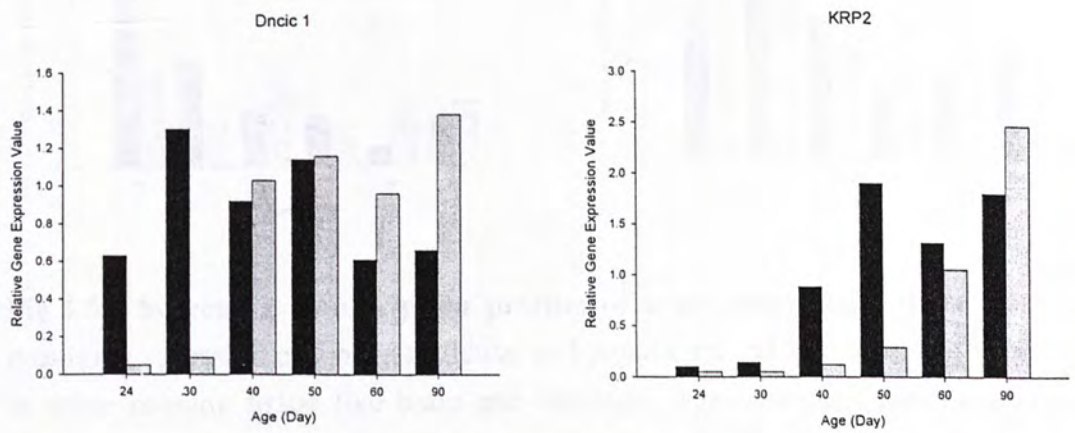


Fig 3.4 Screening transcription profiles of the two motor proteins: Dncic1 and KPR2. KRP2 have the characteristic post-meiotic pattern, while Dncic1 should be of multiple cell origin.

Proteolysis related genes:

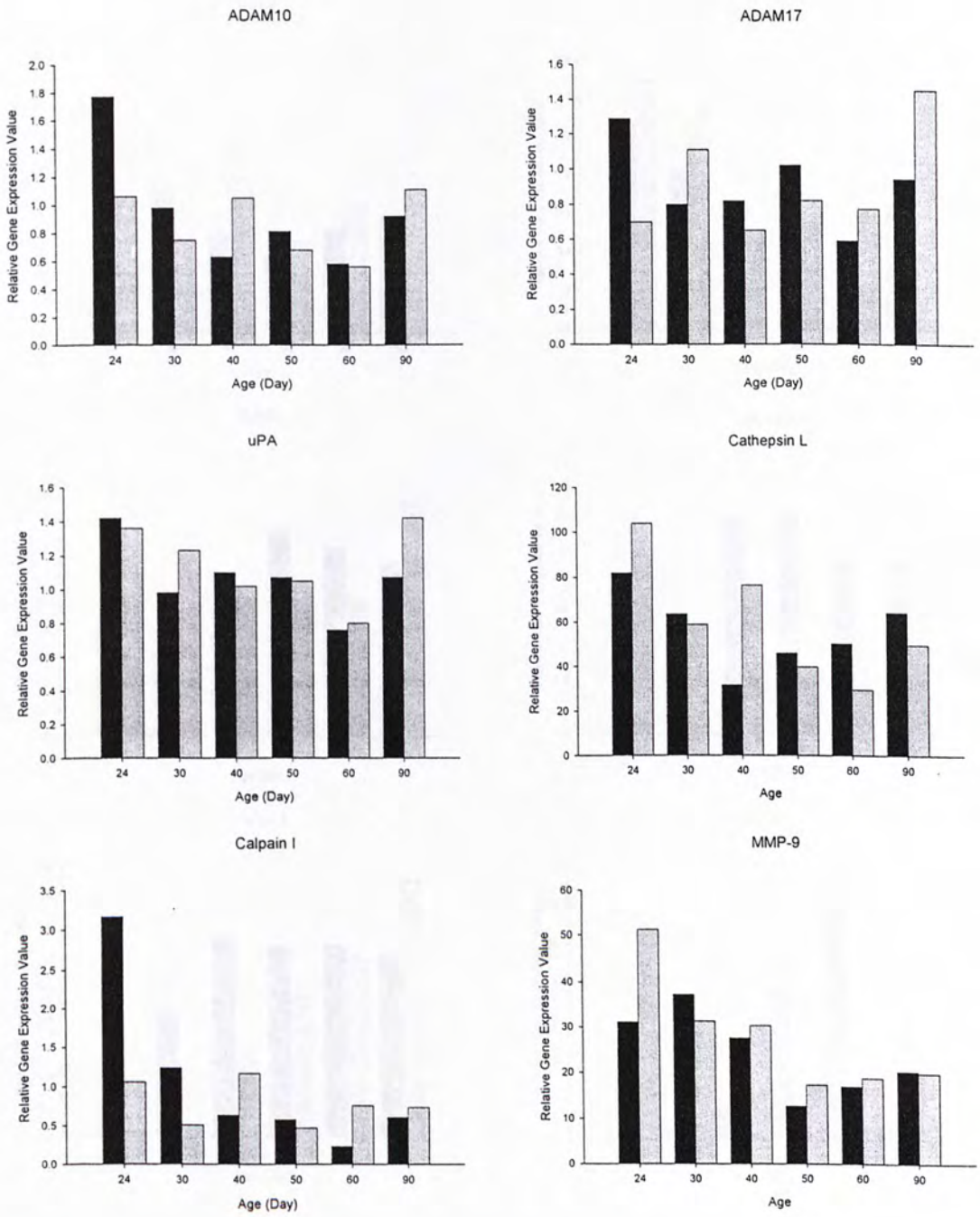


Fig 3.5 Screening transcription profiles of some proteases. All proteases have multiple expression origins in testicular cell population and also abundantly expressed in other somatic tissue like brain and cartilage. Age-dependent down-regulation is possibly suggested in the expression of Calpain I, while the remaining members seem not affected neither by age nor hypothyroid treatment.

Proteases Inhibitors:

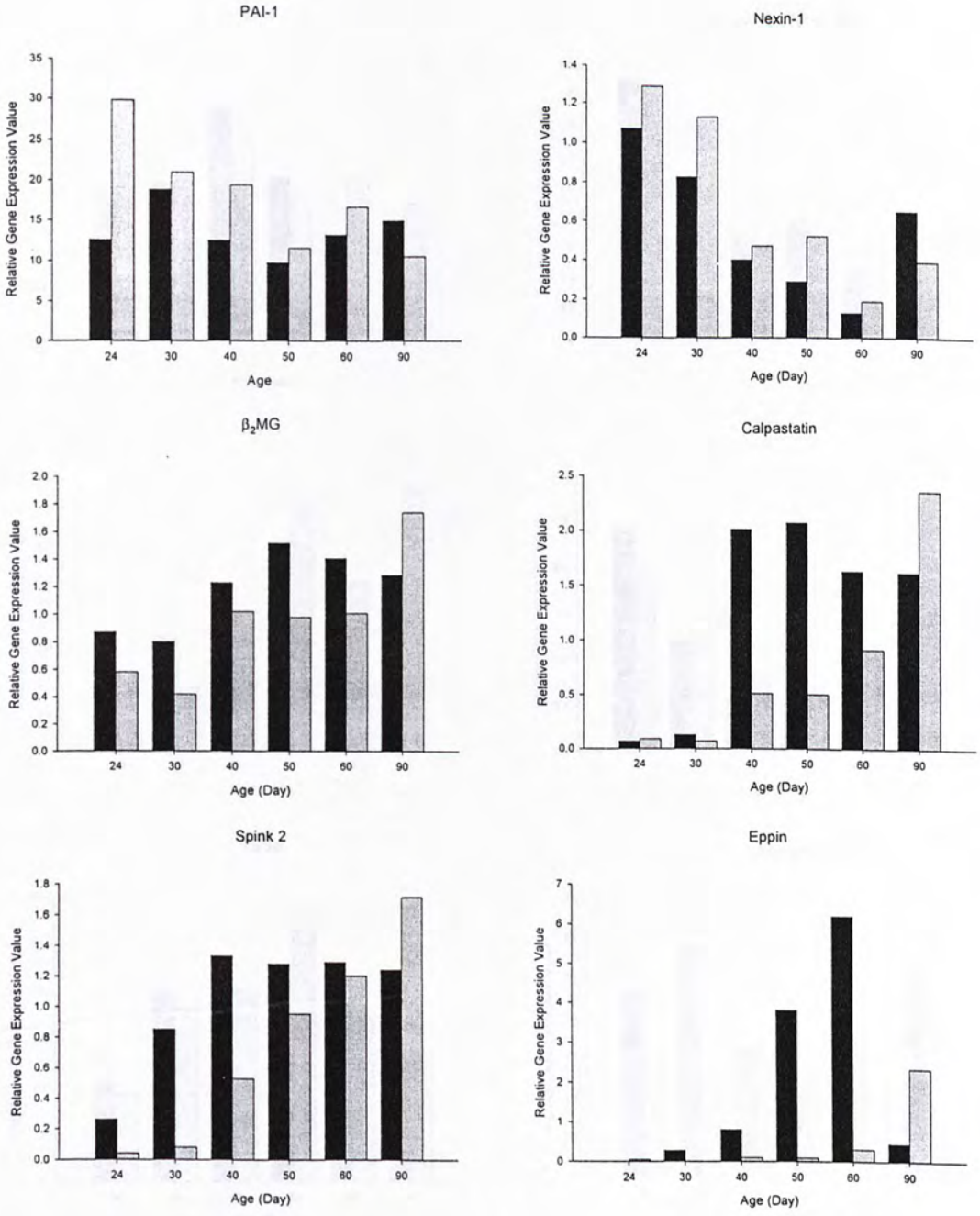


Fig 3.6 Screening transcription profiles of some endogenous protease inhibitors. PAI-1 and β2MG are globally expressed in most of the testicular cells. Nexin-1 is critical in early sex determination and thus higher expression in younger rats is seen. Spink2 seems to be meiotic, while drastic down-regulation of calpastatin and Eppin were shown in adult rats, thus the latter two should be more likely to be post-meiotic.

Junction and Matrix Molecules:

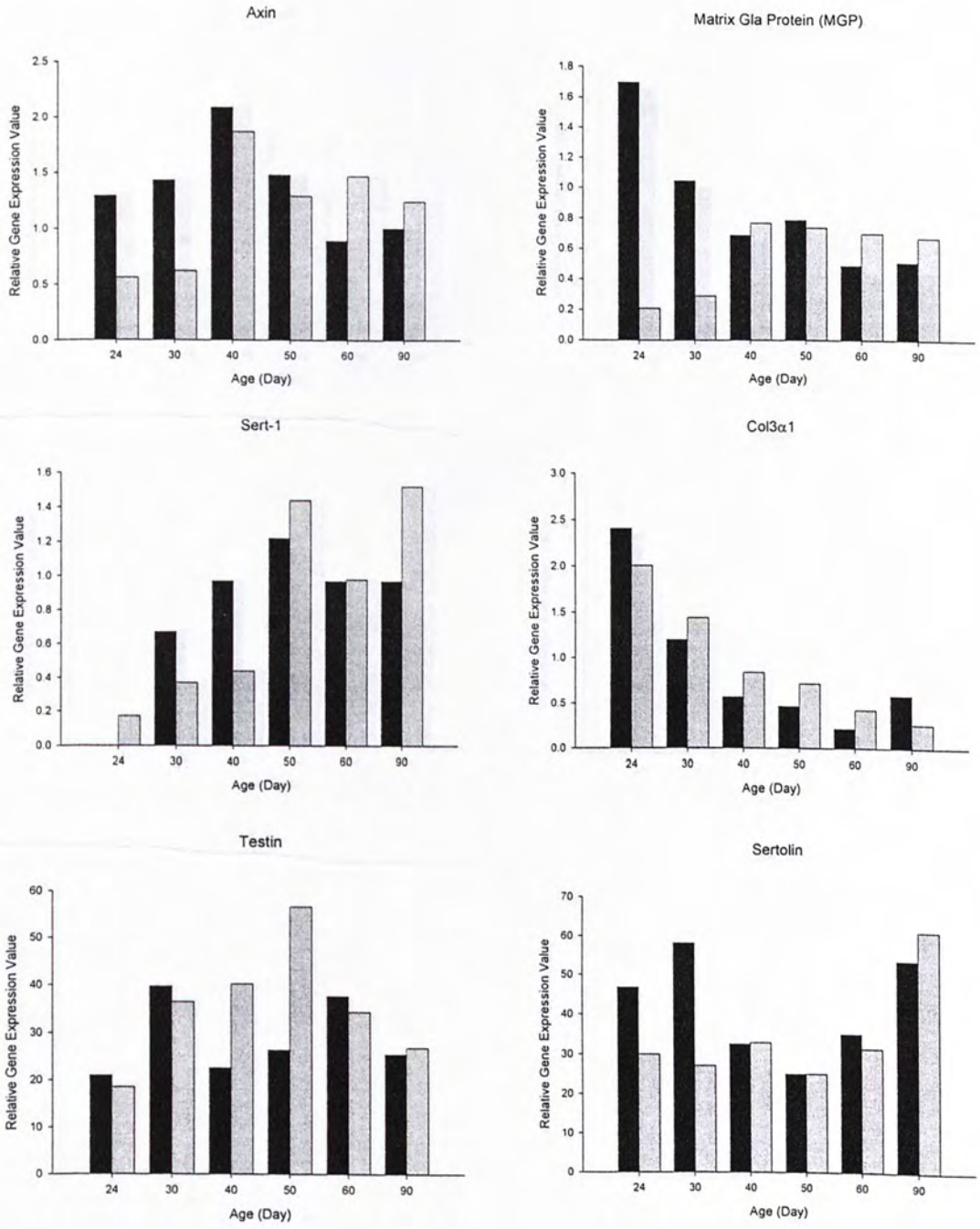


Fig 3.7 Screening transcription profiles of some matrix molecules. MGP and Col3α1 are previously identified as mitotic, Sert-1 as meiotic and testin as somatic. Axin and Sertolin have similar pattern of temporal expression with that of testin, which may suggested that both of them are also widely expressed.

Junction and Matrix Molecules:

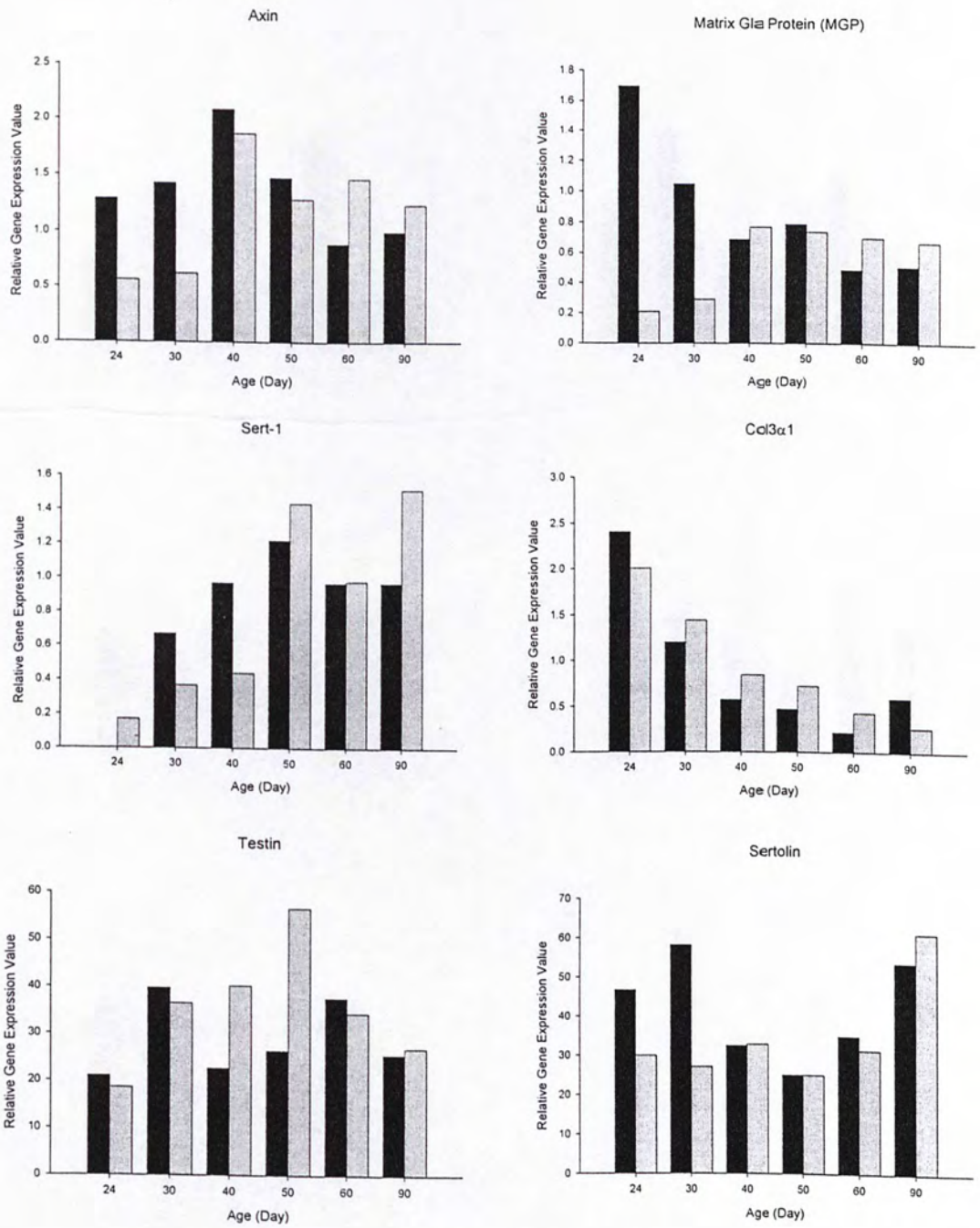


Fig 3.7 Screening transcription profiles of some matrix molecules. MGP and Col3α1 are previously identified as mitotic, Sert-1 as meiotic and testin as somatic. Axin and Sertolin have similar pattern of temporal expression with that of testin, which may suggested that both of them are also widely expressed.

Junction and Matrix Molecules:

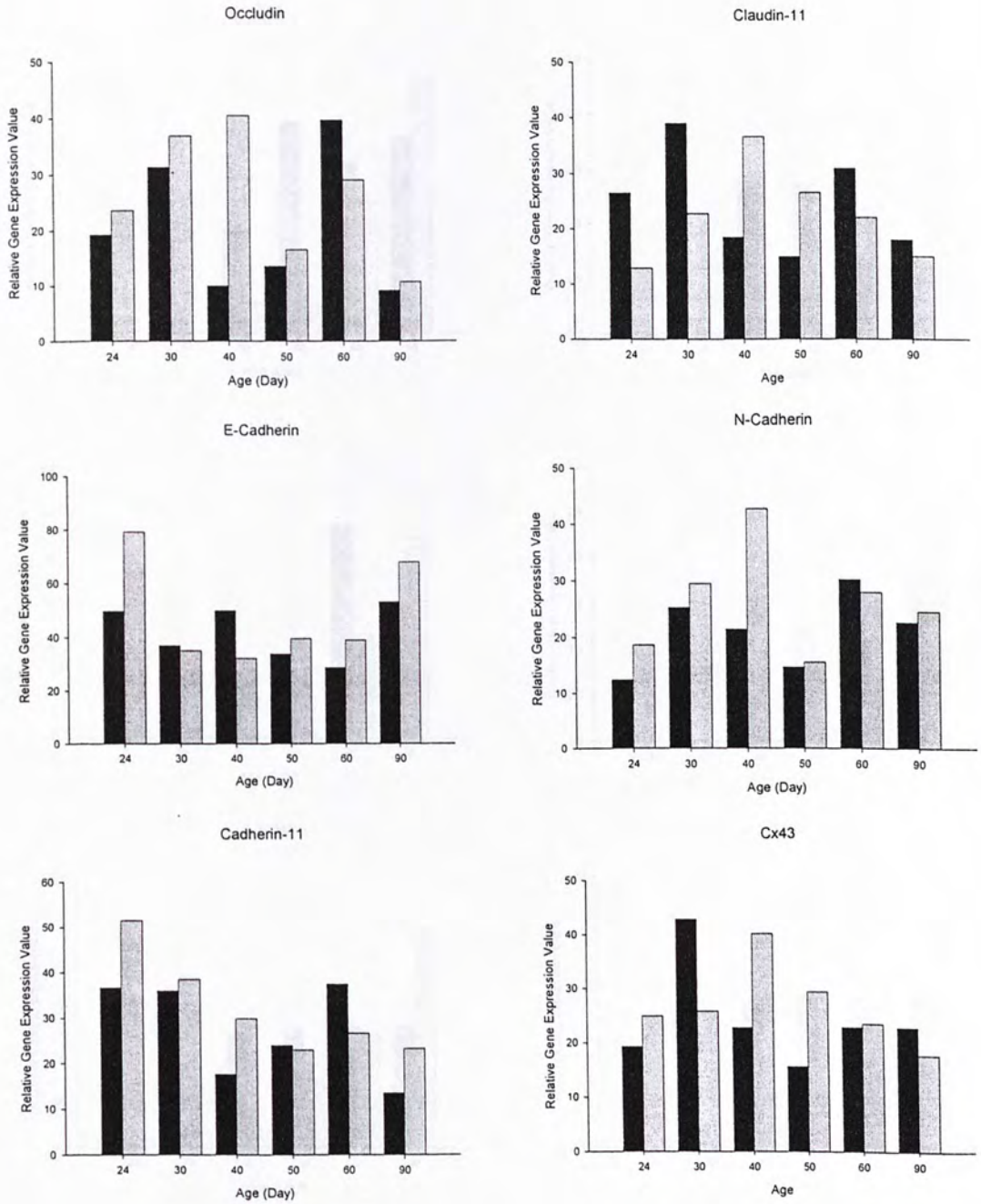


Fig 3.8 Screening transcription profiles of some testicular junction molecules. These six genes are representative members in unique junctions present in testis: tight junction, adhering junction and gap junction. Whether these genes are affected by age or hypothyroid treatments requires further studies, as both Sertoli and germ cells contribute to the total testis expression.

Post-meiotic Spermatids Specific genes:

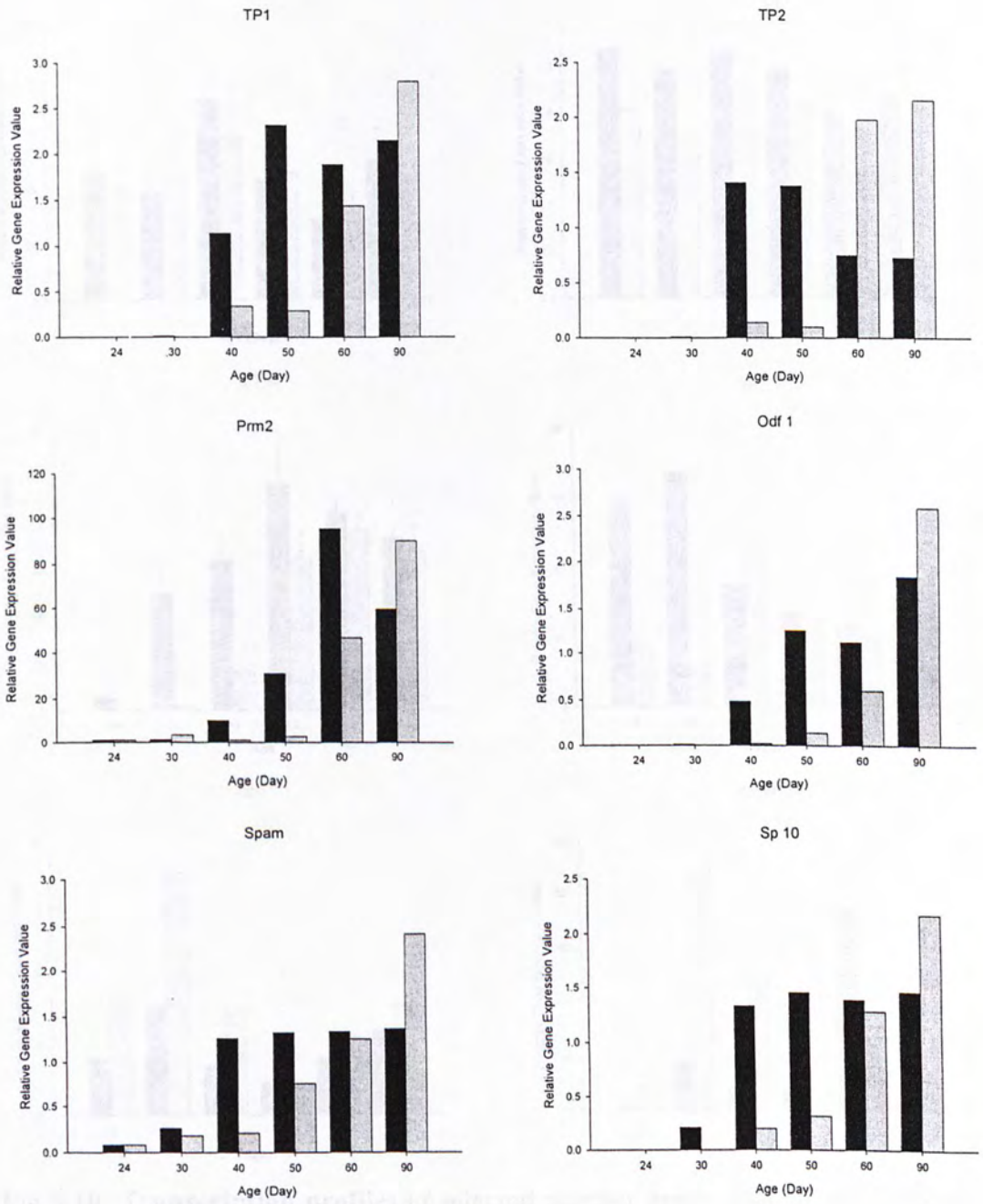


Fig 3.9 Screening transcription profiles of some spermatid-specific genes. TPs and Prms are long-time regarded as markers of post-meiotic spermatids due to their distinctive chromosomal arrangement. Odf1 is also a major constitute of sperm tail, while Sp10 and Spam are related to fertilization and membrane fusion. All of them possess the characteristic Post-Meiotic pattern described in current study.

Cell specific markers:

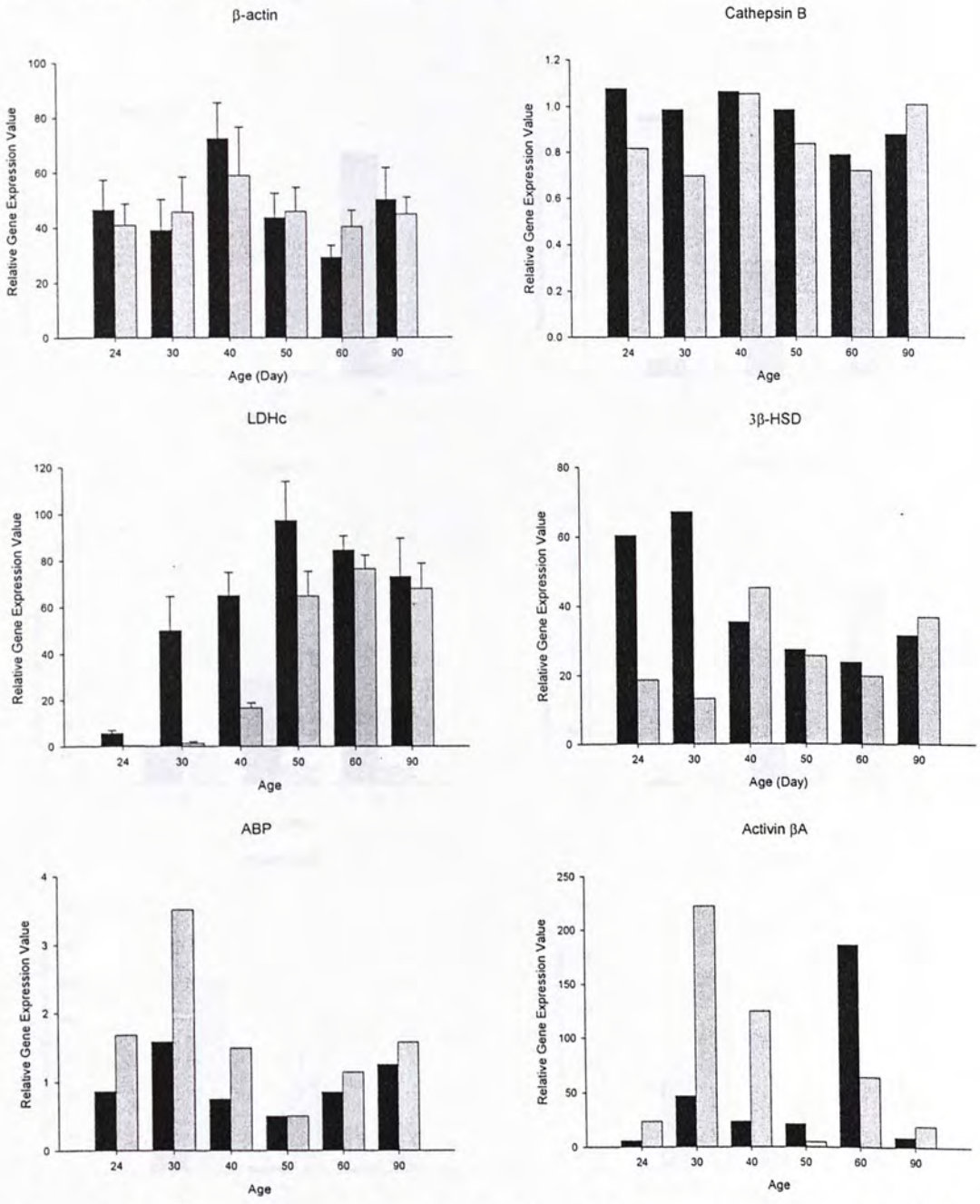


Fig 3.10 Transcription profiles of selected marker genes. β-actin and Cathepsin B has been shown to be universally expressed in all kinds of testicular cells. In our experiment, both of them are not age or treatment affected, thus β-actin has been used as housekeeping gene in my study. LDHc is a marker for meiotic germ cells and possess the typical meiotic pattern. 3β-hydroxysteroid dehydrogenase is specifically expressed in Leydig cells. Androgen binding protein (ABP) and activin βA are markers specifically transcribed in Sertoli cells. Elevated expression under hypothyroidism at early ages suggested the possibility of the presence of proliferating Sertoli in this period.

Expression of non-spermatogenic genes at neonatal age

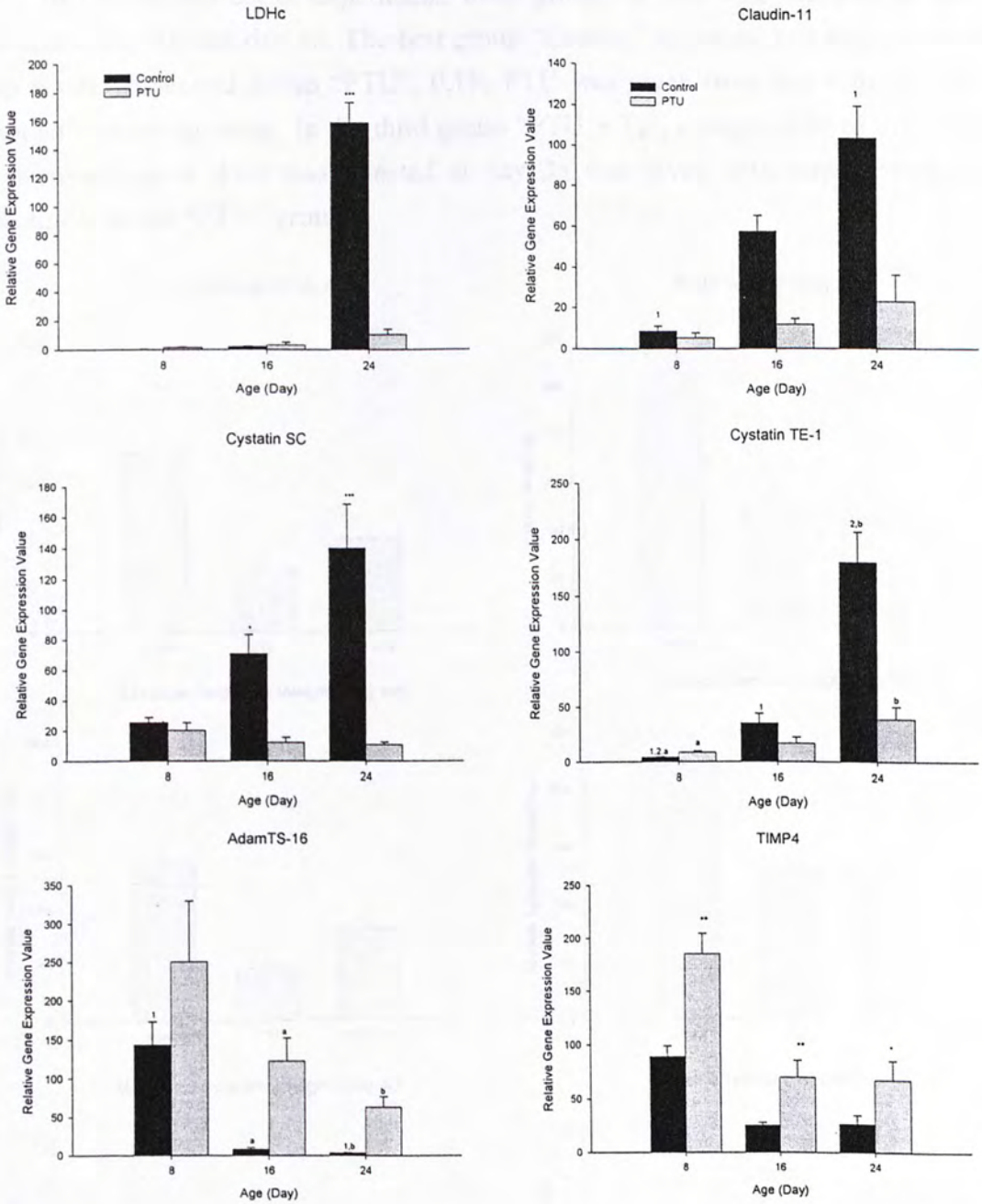


Fig 4: Expression of selected genes under normal and hypothyroid condition before puberty. In this experiment, hypothyroidism was induced by adding 0.05% MMI in mother rats' drinking water from prenatal day 16 until scarified. Expression of all genes above are significantly affected by age, treatment and their interaction after two-way AVONA with $p>0.01$. Annotations above columns denotes for statistically higher expression by Dunn's test with different significance. ** denotes for $p<0.01$, * for $p<0.05$.

Responsiveness of gene transcription after thyroxin Replacement

In this second set of experiment, three groups of rats were scarified at age of postnatal day 40 and day 50. The first group "Control" is normal rats that given with tap water. In second group "PTU", 0.1% PTU was given from day 4 to day 24 in mother's drinking water. In the third group "PTU + T_4", a single dose of thyroxin at sub-physiological dose was injected at day 35 that given with same hypothyroid treatment as the "PTU" group.

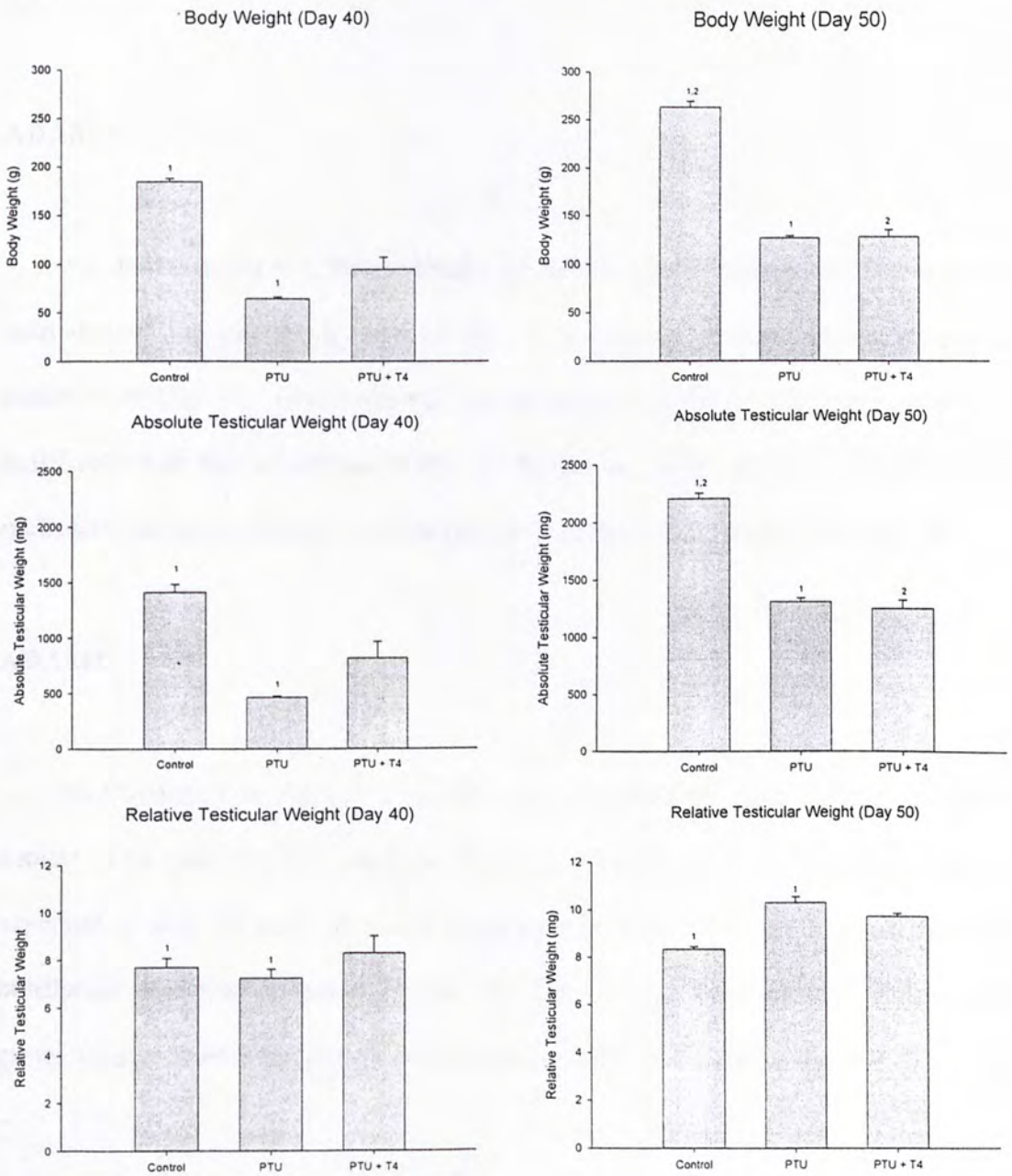


Fig 5.1 Body and testis weights (per paired testes) in second set of experiment with T_4 replacement therapy. Significant differences revealed by Dunn's test are shown as number pairs on top of columns.

Body and testis weights

In this set of experiment, comparable results can be found in control and PTU groups as before as shown in Fig 5.2. T_4 replacement cannot restore body, absolute and relative testis weight changes by the neonatal hypothyroidism at both day 40 and 50.

ADAM2:

As shown in Fig 5.2, the expression of ADAM2 under hypothyroidism is similar with that of the previously descript (Fig 2.1). Significant down-regulation can be observed at Day 40 under neonatal hypothyroidism ($p<0.01$), while transcription is indifferent with that of normal at Day 50. Single dose of T_4 replacement at day 35 is ineffective to restore the lowered transcription levels to PTU treatment at day 40.

ADAM3:

As illustrated in Fig 5.3, the expression of ADAM3 under hypothyroidism is similar with previous descriptions (Fig 2.2). Significant down-regulation can be observed at Day 40 under neonatal hypothyroidism ($p<0.05$), while transcription is indifferent with that of normal at Day 50. Due to large variation in T_4 replacement group, only a trend of transcription recovery ($p<0.19$) was found at day 40.

ADAM4:

The responsiveness of T4 replacement of ADAM4 is shown in Fig 5.4. Previous experiment showed ADAM4 suffered a prolonged down-regulation under neonatal hypothyroidism (Fig 2.3) when compared with ADAM2, 3 and 5. (Fig 2.1, 2.2 and 2.4 respectively). Again, expression of ADAM4 in PTU-treated rats is significantly lowered than that of control at both day 40 and 50 in this study ($p<0.01$ and <0.001 respectively). T_4 replacement is able to rescue the decreased expression levels at day 40 to certain extent but not back to the normal level ($p<0.14$). At day 50, both PTU and T_4 replacement groups are significantly decreased ($p<0.001$) when compared to the control rats.

ADAM5:

As shown in Fig 5.5, a severe down-regulation of ADAM5 is observed at both day 40 and day 50 as before (Fig 2.4). Unlike ADAM2, T_4 replacement restores target gene transcription to normal level at day 40, but not at day 50. Number above columns revealed statistical significance by Dunn's test after two-way ANOVA analysis with $p<0.05$

ADAM6:

Similar with that of ADAM4, a comparable down-regulation pattern is also observed in case of ADAM 6 which is illustrated in Fig 5.6. Significantly lowered transcriptions are observed at both day 40 to 50 ($p<0.01$ and <0.001 respectively). Sharing the similarities with that of ADAM 4, T_4 replacement is only effective to

recover the lowered expression to certain extent bur not significantly back to normal at day 40 ($p<0.136$). At day 50, same result of ADAM4 is achieved.

ADAM18:

As shown in Fig 5.7, a severe down-regulation of ADAM18 is observed at day 40 ($p<0.001$) but not at day 50 as before (Fig 2.7). Again due to the large variation of T_4 replacement group, only a trend of recovery ($p<0.06$) can be seen at day 40, but no statistical difference can be found at day 50.

Pcsk4:

Transcriptions of Pcsk 4 in this study are similar to the results of ADAM5. With decreased expression upon hypothyroid treatment ($p<0.05$ at both age), T_4 replacement is able to restore the transcription of this gene back to normal at day 40 ($p<0.05$) but not at day 50.

PRSS21:

Another putative protease, PRSS21, have shown similar responsiveness (Fig 5.9) with that of ADAM2 (Fig 5.2). Hypothyroid-induced down-regulation was previously shown (Fig 2.17) to be effective only before day 40 ($p<0.05$), and expression recovered after day 50. Same observations can be seen in this set of experiment, and also revealed that T_4 replacement recover the lowered transcription due to PTU treatment at day 40 ($p<0.05$). However, this recovery is not extended up to day 50.

ADAMTS-16:

Just like the transcriptions of ADAM4 (Fig 5.4) and ADAM6 (Fig 5.6), significant down-regulation was observed under hypothyroidism even until day 50 ($p<0.001$), which is demonstrated in Fig 5.10. The administration of single dose of T_4 is able to rescue the transcription levels to a higher but not back to the normal levels at Day 40 ($p<0.632$). At day 50, both PTU and T_4 replacement group are significantly lowered in their expression of ADAMTS-16 when compared to their control group ($p<0.001$).

TIMP-4:

Similar trends can be observed in protease inhibitors like TIMP-4 (Fig 5.11). Hypothyroid rats suffer from significant down-regulation at day 40 and day 50 ($p<0.05$). Thyroxin replacement was able to restore certain degree of transcription reduction due to hypothyroidism ($p<0.38$ when compared with control), but not in day 50 ($p<0.05$ when compared with control).

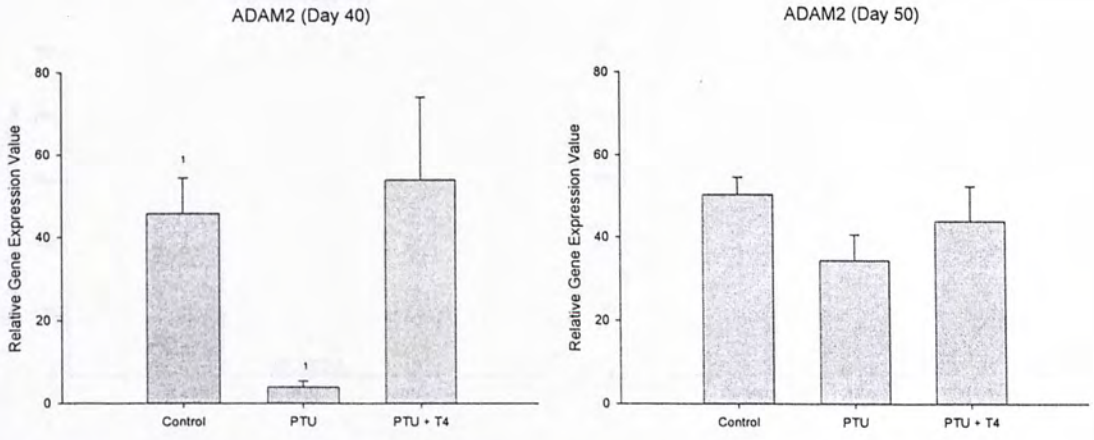


Fig 5.2 Response of ADAM2 gene expression at day 40 and 50 under T_4 replacement.

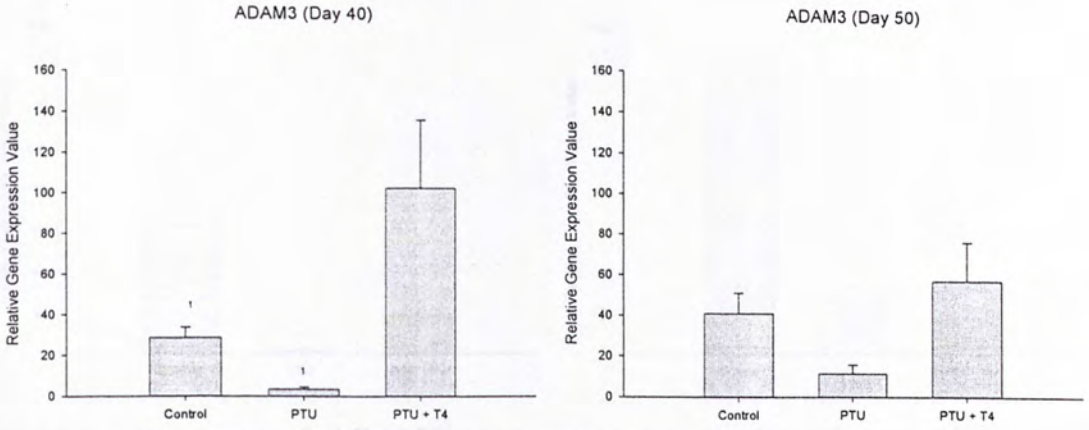


Fig 5.3 Response of ADAM3 gene expression at day 40 and 50 under T4 replacement.

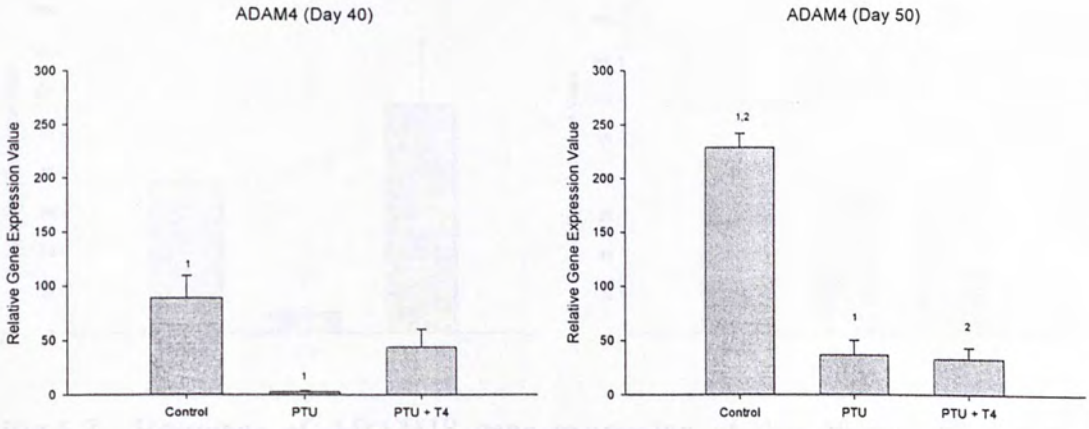


Fig 5.4 Response of ADAM4 gene expression at day 40 and 50 under T_4 replacement.

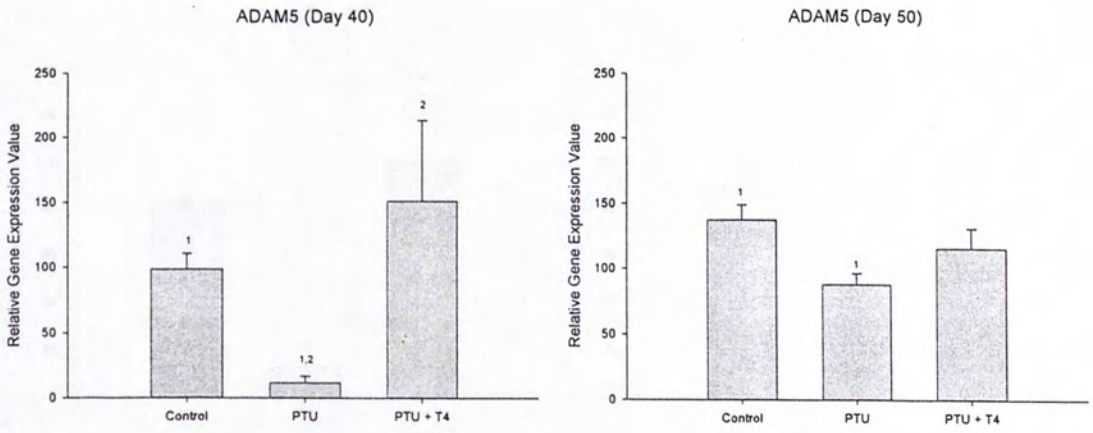


Fig 5.5 Response of ADAM5 gene expression at day 40 and 50 under T_4 replacement.

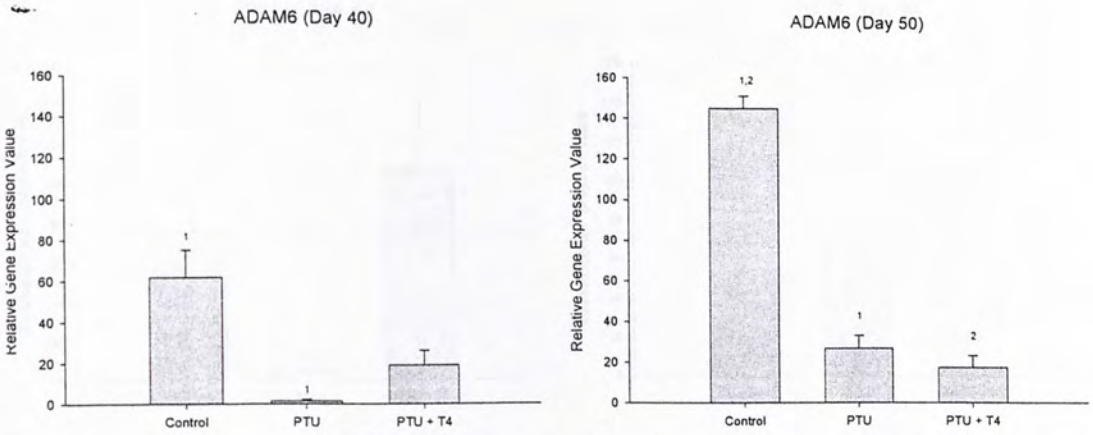


Fig 5.6 Response of ADAM6 gene expression at day 40 and 50 under T_4 replacement.

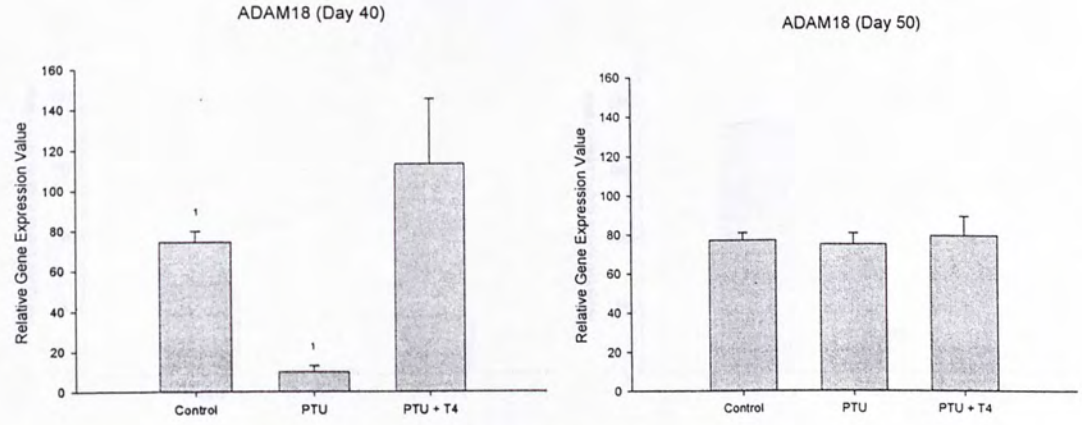


Fig 5.7 Response of ADAM18 gene expression at day 40 and 50 under T_4 replacement.

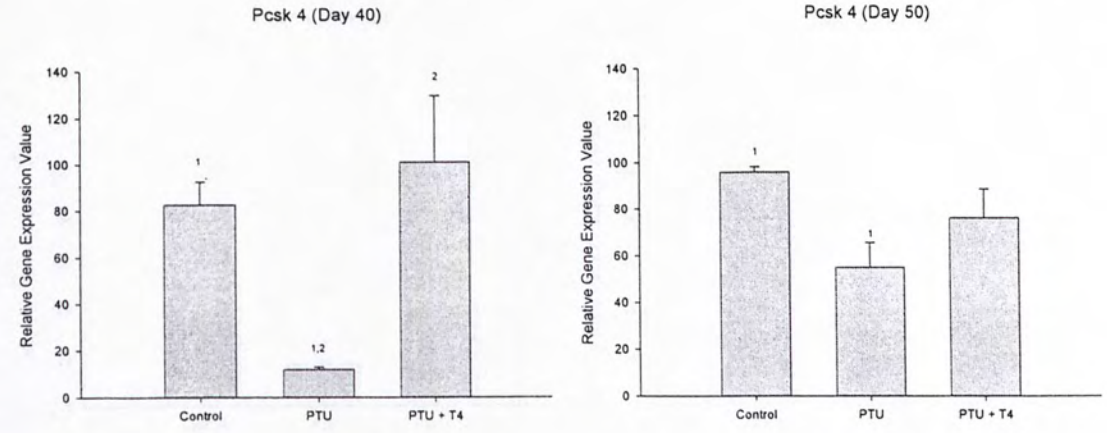


Fig 5.8 Response of Proprotein convertase subtilisin/kexin type 4 (Pcsk4) gene expression at day 40 and 50 under T_4 replacement.

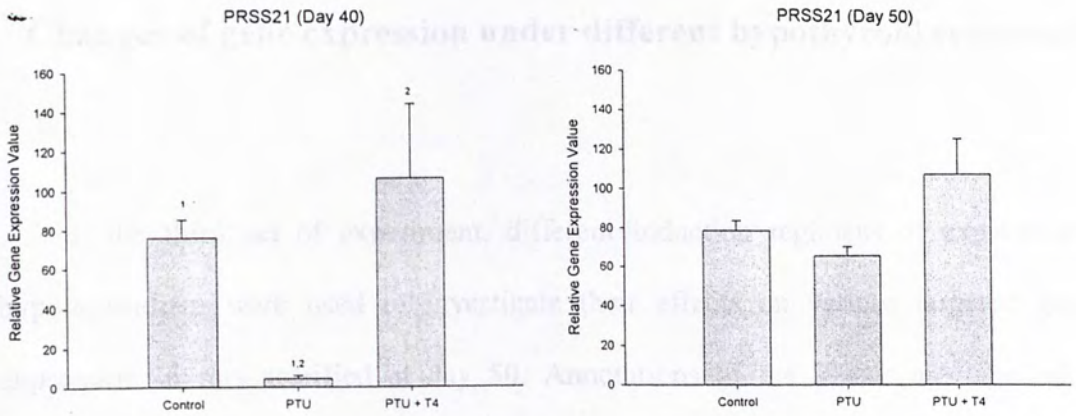


Fig 5.9 Response of PRSS21 gene expression at day 40 and 50 under T_4 replacement.

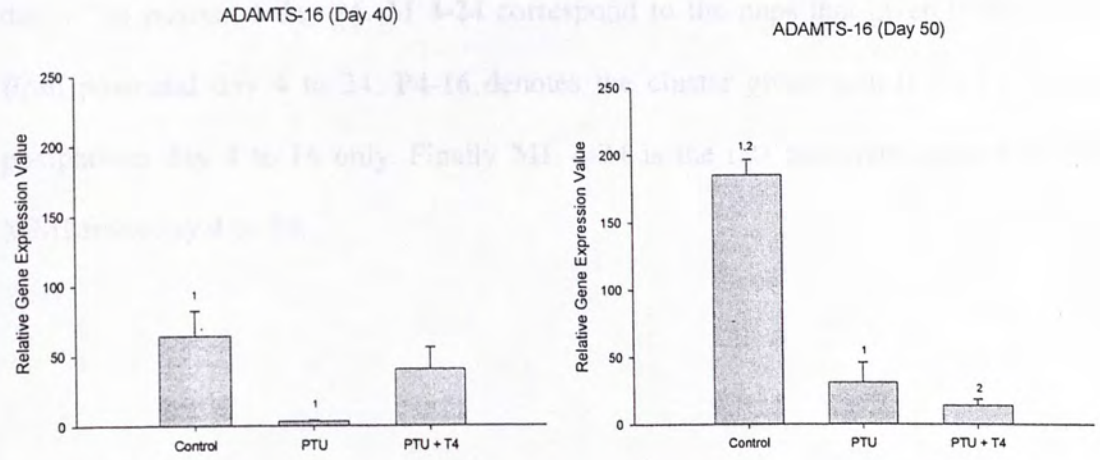


Fig 5.10 Response of ADAMTS-16 gene expression at day 40 and 50 under T_4 replacement.

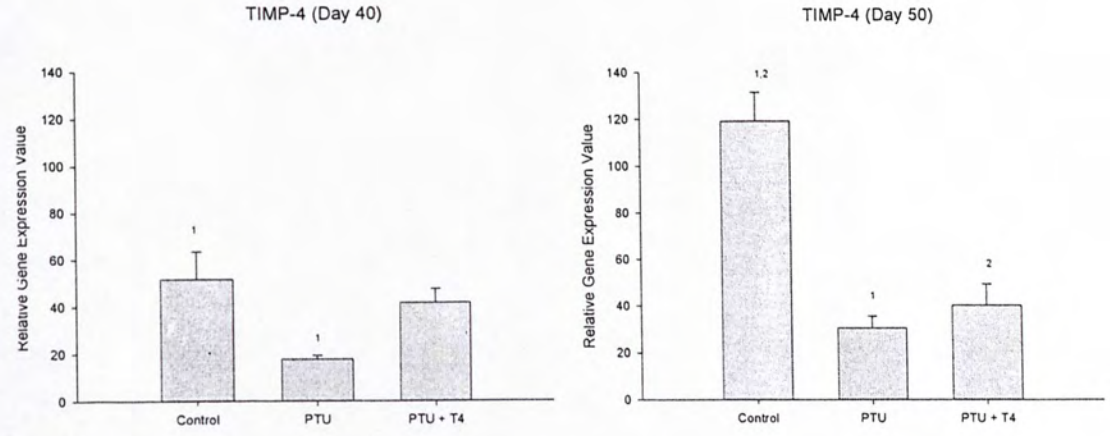


Fig 5.11 Response of TIMP-4 gene expression at day 40 and 50 under T_4 replacement.

Changes of gene expression under different hypothyroid regimens

In the third set of experiment, different induction regimens of experimental hypothyroidism were used to investigate their effects on various targeted genes expression in rats scarified at day 50. Annotations of the X-axis are labelled as follows: **P4-24** represent the group of rats that given with 0.1% PTU from postnatal day 4 to 24. **M p17-26** stands for the group that start the 0.05% MMI from prenatal day 17 to postnatal day 26. **M 4-24** correspond to the pups that given 0.05% MMI from postnatal day 4 to 24. **P4-16** denotes the cluster given with 0.1% PTU from postpartum day 4 to 16 only. Finally **ML 4-24** is the rats that were given 0.0125% MMI from day 4 to 24.

Body Weight:

The change in body weight under regimens of hypothyroidism is demonstrated in Fig 6.1. Except the group that was given with lowered MMI concentration, all other treatment of hypothyroidism caused significant decrease in body weight at the levels of $p<0.001$. Shortening the period of PTU administration by 8 days returns total body weights of rats significantly ($p<0.001$). The group of 0.0125% MMI group is not suffered from decreased body weight when compared with other treatments ($p<0.01$)

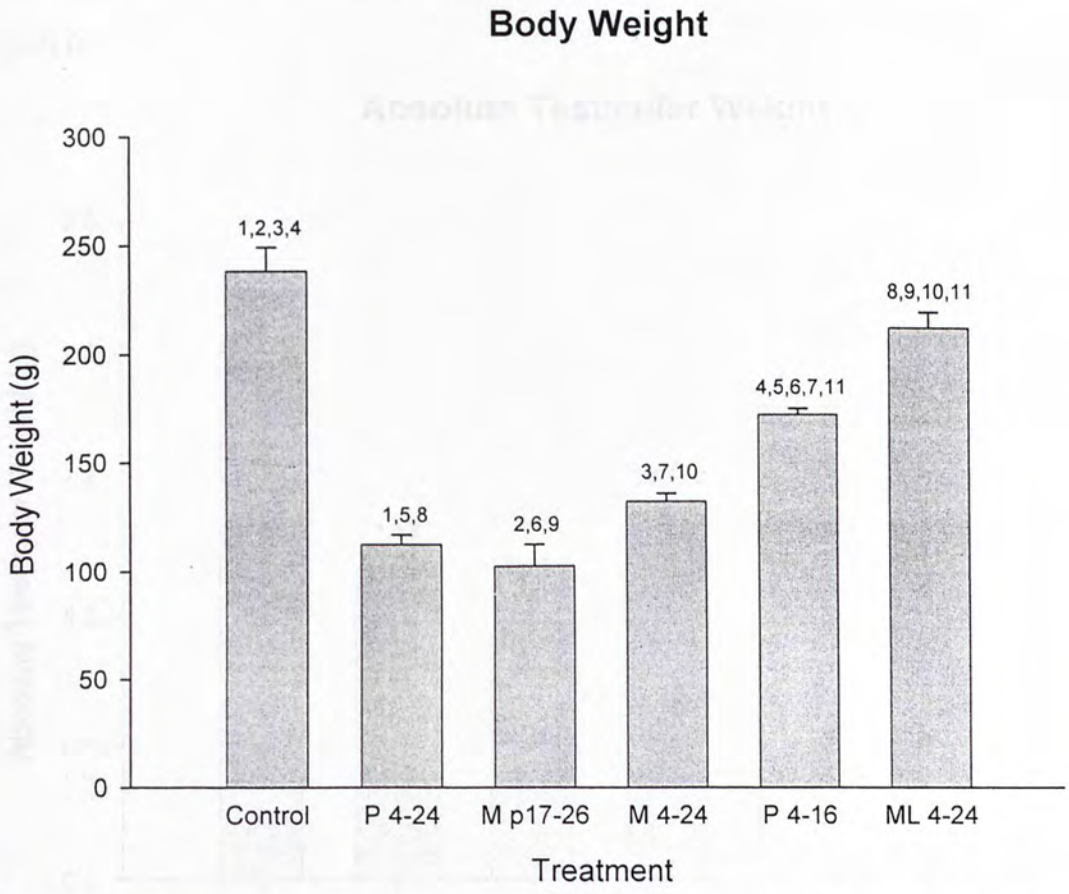


Fig 6.1 Alternations in body weight under different induction regimens of hypothyroidism. Number pairs on each column represent statistical differences revealed by Dunn's test after one-way ANOVA with $p<0.05$.

Absolute testicular weight:

The change in absolute testicular weight is summarized in Fig 6.2. When comparing testis weights of rats, the first three groups of treated rats cannot restore their testis weight loss ($p<0.001$), while the groups that benefits from shortened PTU administration and lowed MMI concentration have comparable testis size with that of normal. Ending the PTU treatment at day 16 can recover most of the testis weight loss when compared with that ends at day 24 ($p<0.01$). Pups with prolonged MMI administration before birth suffered a further decrease in absolute testis weight than with postnatal treatment only ($p<0.05$). Again, pups with 0.0125% MMI administration have significant larger testis than the MMI treatments at 0.05% ($p<0.001$).

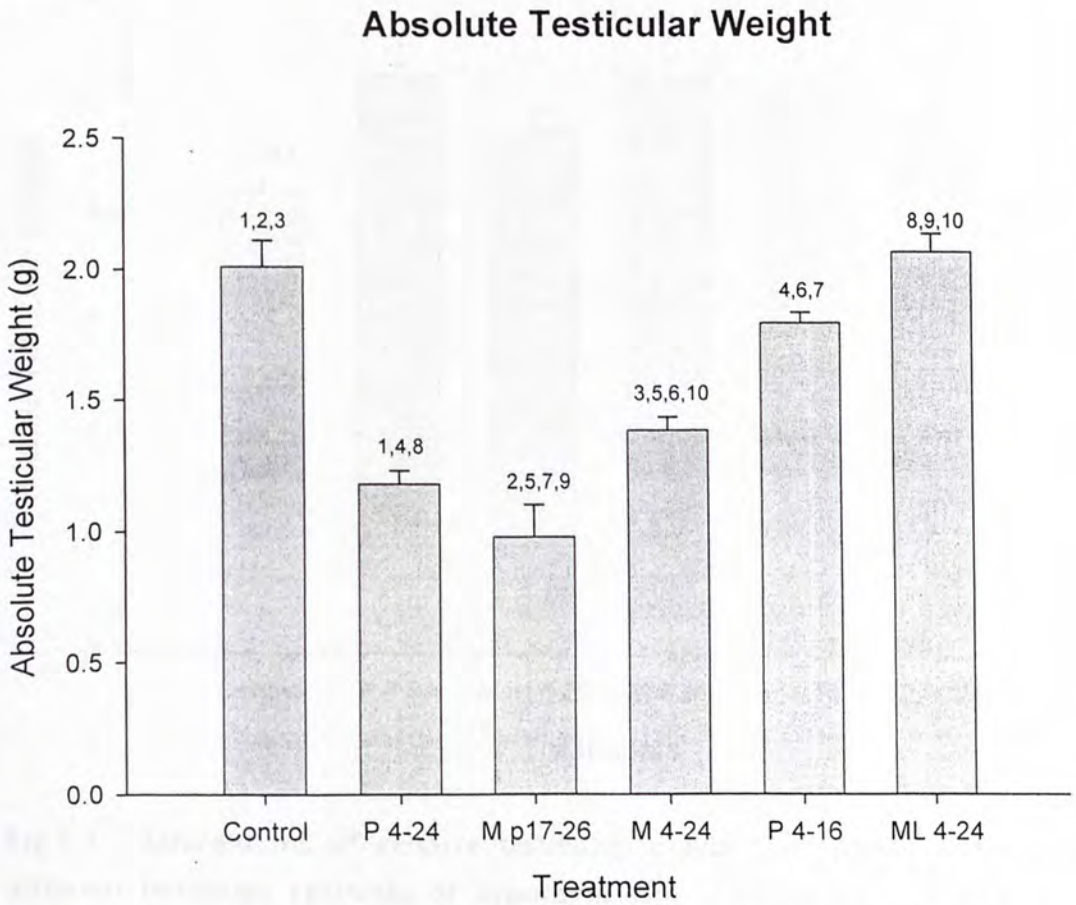


Fig 6.2 Alternations of absolute testicular weight (per paired testes) under different induction regimens of hypothyroidism. Number pairs on each column represent statistical differences revealed by Dunn's test after one-way ANOVA.

Relative testicular weight:

Interesting findings are found in term of relative testis weights in this study which is illustrated in Fig 6.3. All treatment groups except the one with extended hypothyroid period have significant larger relative testes weights. However, this increase in relative testicular size is more or less at the same levels, no significances were found among these groups. Number pairs on each column represent statistical differences revealed by Dunn's test after one-way ANOVA with $p<0.05$.

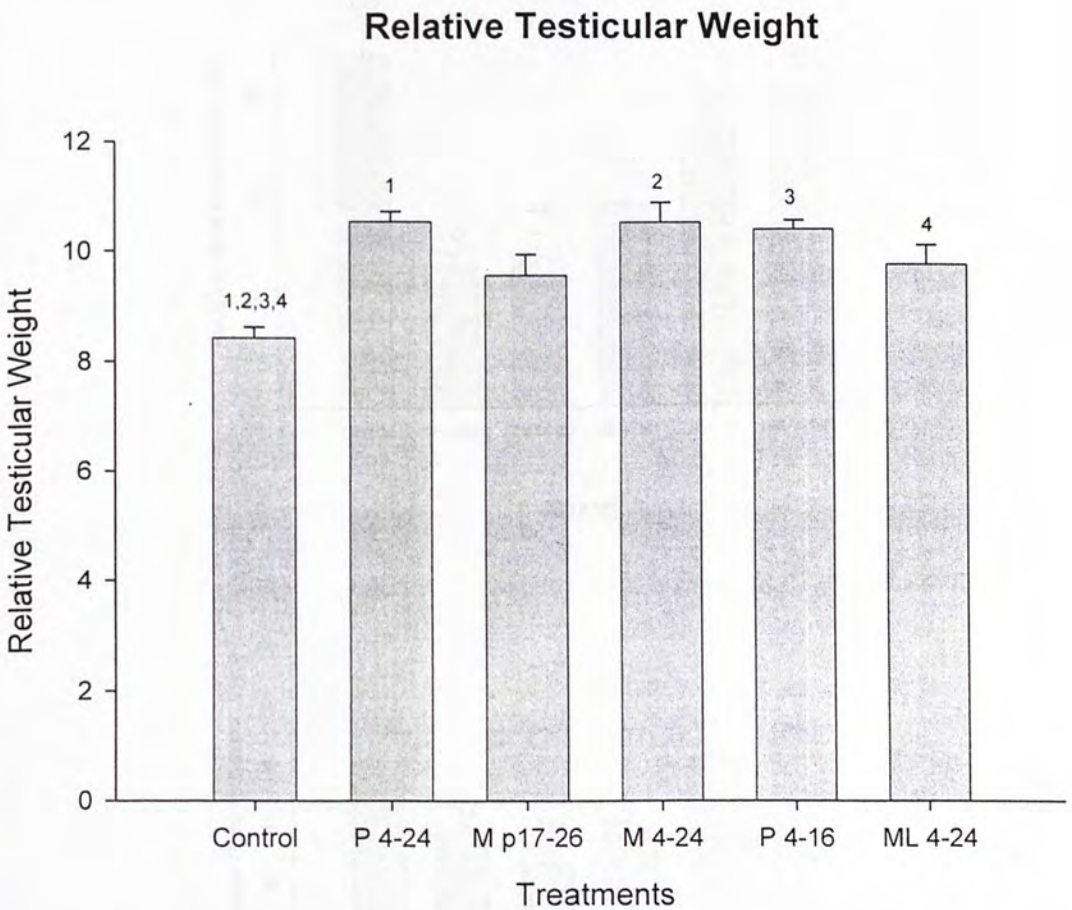


Fig 6.3 Alternations of relative testicular weight (per paired testes) under different induction regimens of hypothyroidism. Number pairs on each column represent statistical differences revealed by Dunn's test after one-way ANOVA with $p<0.05$.

ADAM2 and ADAM5:

As summerized in Fig 6.4, a significant downregulation of both genes can be identified at 0.1% PTU as demonstrated before (Fig 2.1 and 2.4 respectively. Significant reduction of transcription can also be observed at groups with MMI treatment under the prolonged and standard duration of hypothyroid induction.). No significant down-regulations were seen in groups that neither treated with shortened period nor lowered concentration of MMI.

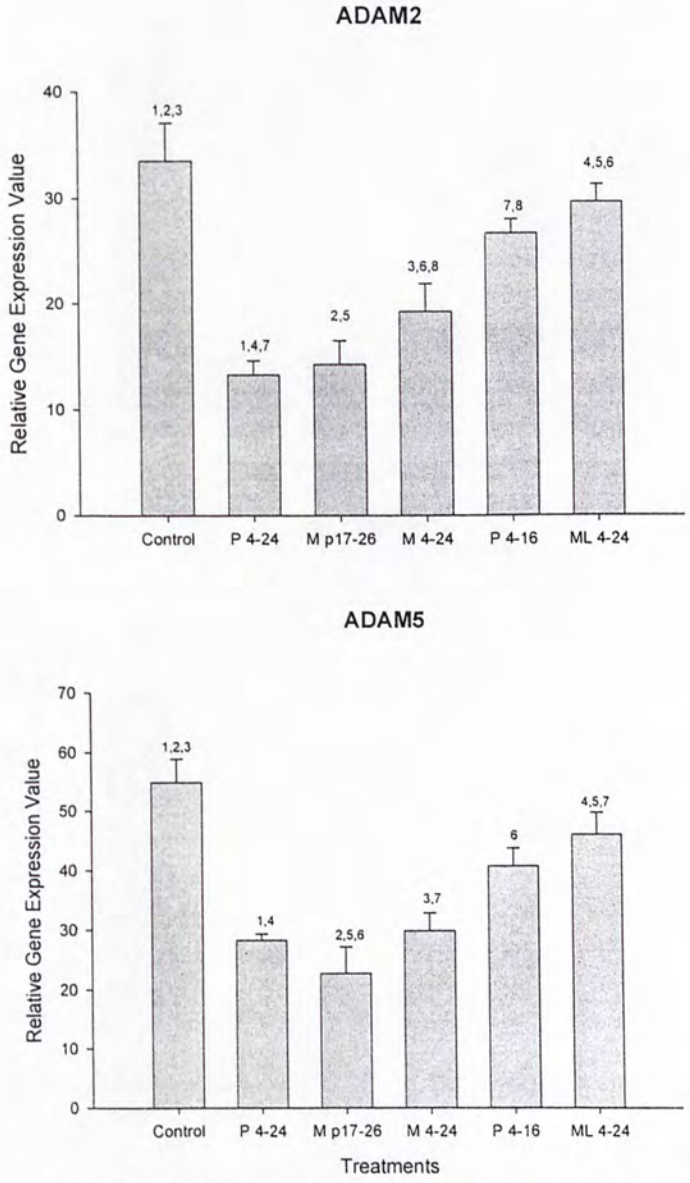


Fig 6.4 Transcriptions of ADAM2 and ADAM5 genes under different neonatal hypothyroid regimens. All number pairs on each column revealed statistical differences with $p<0.05$ by Dunn's test.

ADAM6 and ADAMTS-16:

As illustrated in Fig 6.5, severe down-regulation can be observed in all treatments except the one fed with lower MMI concentration. Major down-regulations are observed under normal PTU and prolonged MMI treatment ($p<0.001$), with a less severe decrease in the other two groups.

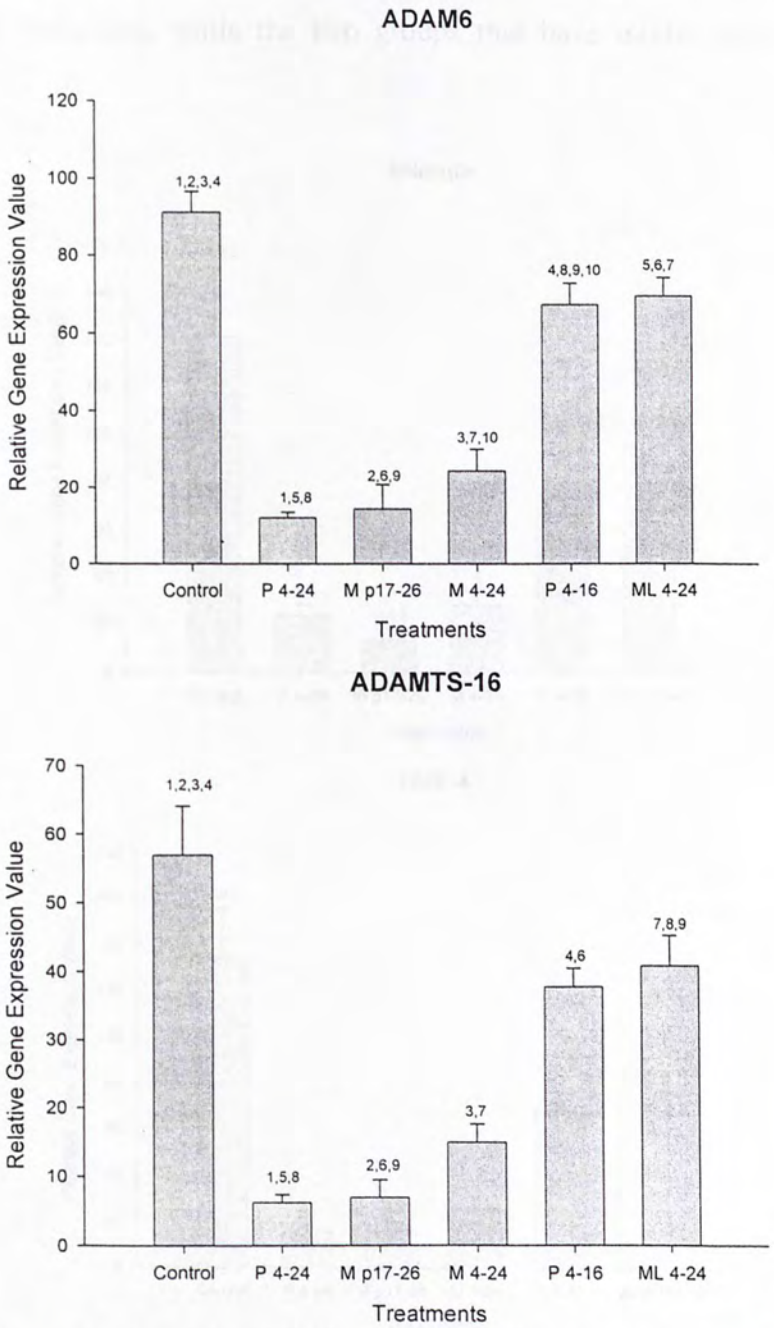


Fig 6.5 Transcriptions of ADAM4 and ADAM6 genes under different neonatal hypothyroid regimens. All number pairs on each column revealed statistical differences with $p<0.05$ by Dunn's test.

Bikunin and TIMP-4:

As demonstrated in Fig 6.6, these two endogenous protease inhibitors have similar expression pattern under different induction regimen. Again, significant down regulations were seen on the first three groups that applied with more intense hypothyroid induction, while the two groups that have milder treatments are not affected.

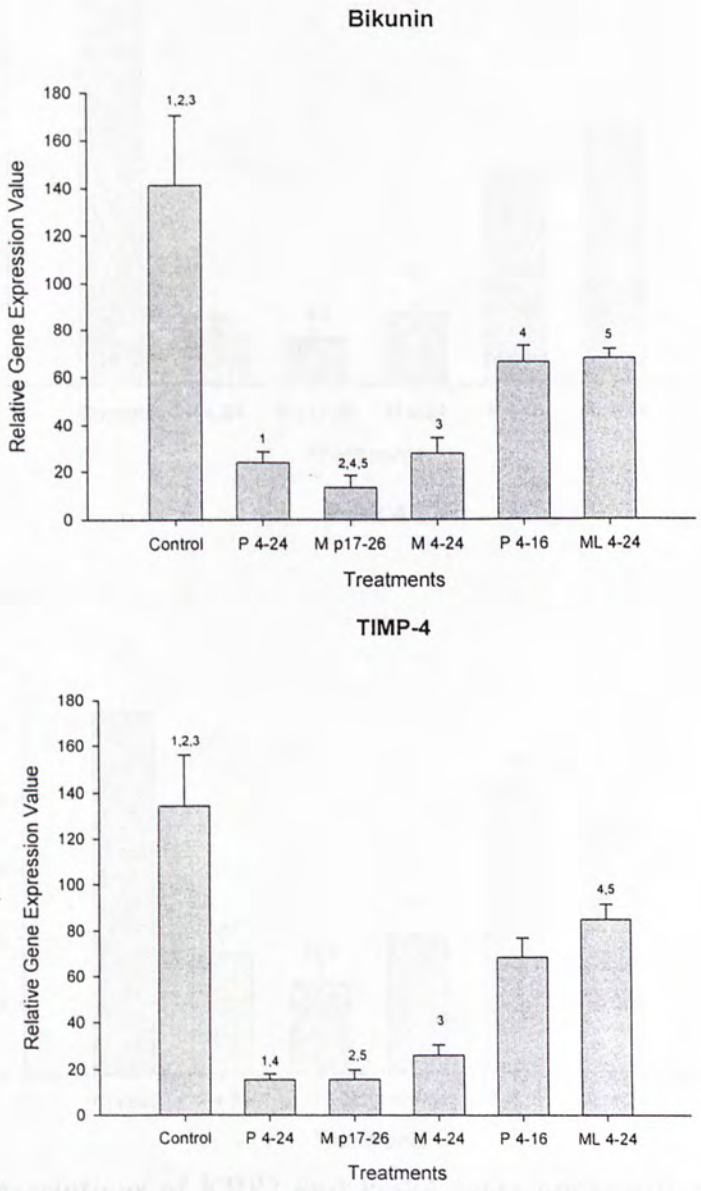


Fig 6.6 Transcriptions of Bikunin and TIMP-4 under different neonatal hypothyroid regimens. Number pairs revealed statistical differences with $p<0.05$ by Dunn's test.

KRP2 and Pcsk4:

As shown in Fig 6.7, expressions were decreased significantly and equally under PTU and MMI administration for normal and extended period, while shortened or milder goitrogens treatment groups are not affected.

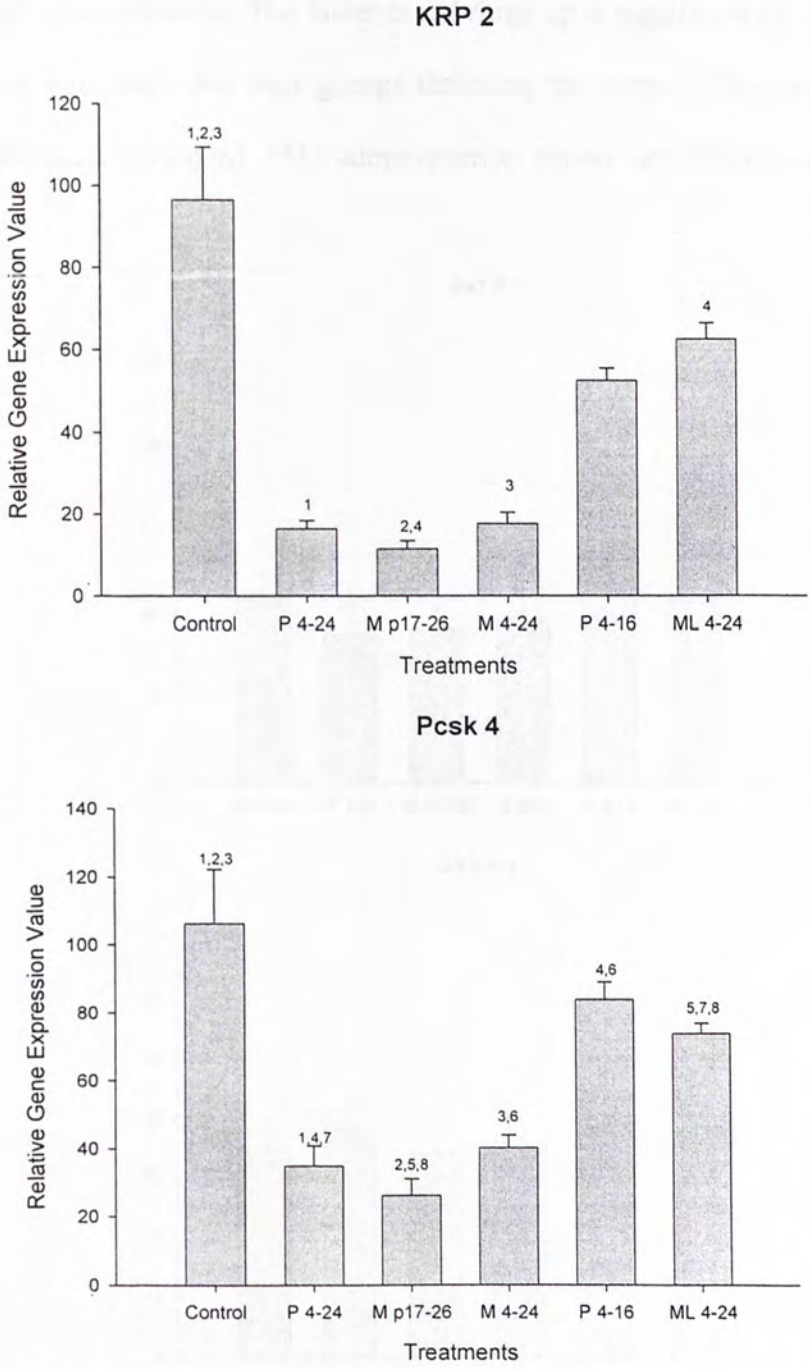


Fig 6.7 Transcriptions of KRP2 and Pcsk4 genes under different hypothyroid regimens. All number pairs on each column revealed statistical differences with $p<0.05$ by Dunn's test.

Pkiβ and GATA-4:

In contrast with the previous genes, the mitotic Pkiβ and GATA-4 display a completely different picture to the hypothyroid responses as shown in Fig 6.8. Expression of both genes is similar in all treatment groups except the one given with low MMI concentration. The latter case brings up a significant up regulation when compared with the other four groups including the control. The group that applied with shortened period of PTU administration shows no differences with all other groups

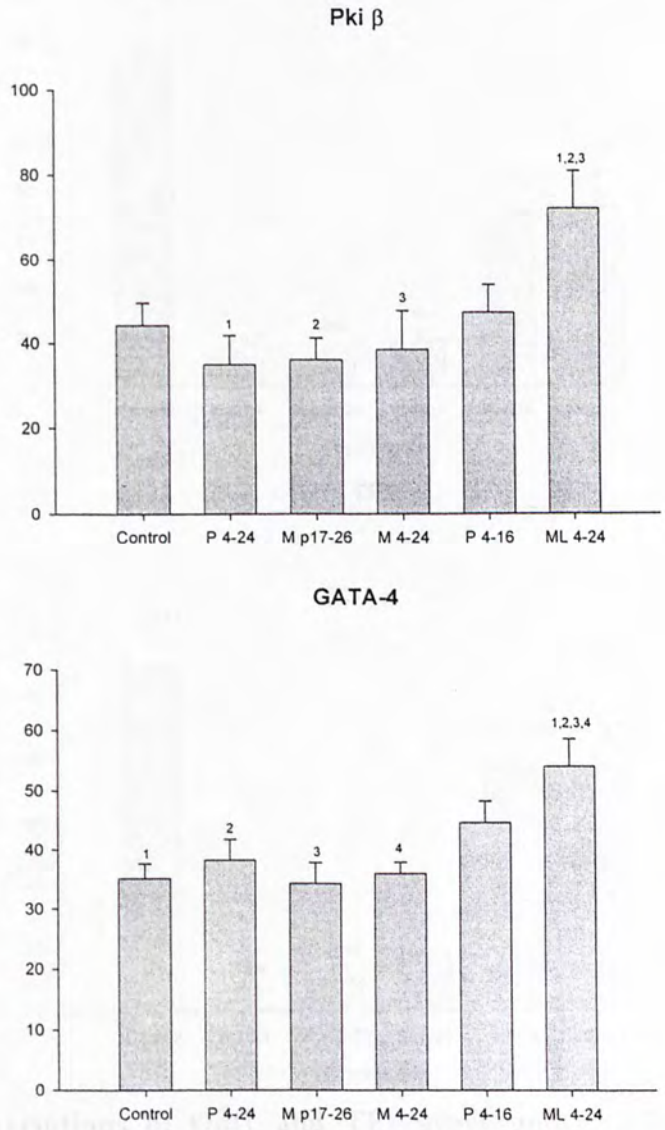


Fig 6.8 Transcriptions of Pkiβ and GATA-4 genes under different hypothyroid regimens. Number pairs revealed statistical differences with $p<0.05$ by Dunn's test.

Odf1 and TP1:

Expressions of spermatid-specific genes are quite unique in this study as demonstrated in Fig 6.9. All treatments suffered from different degrees of down-regulation of Odf1 and TP1 under hypothyroidism. The degree of down-regulation depends on the intensity of goitrogen treatment, where the effect on two less intensive groups is relatively smaller.

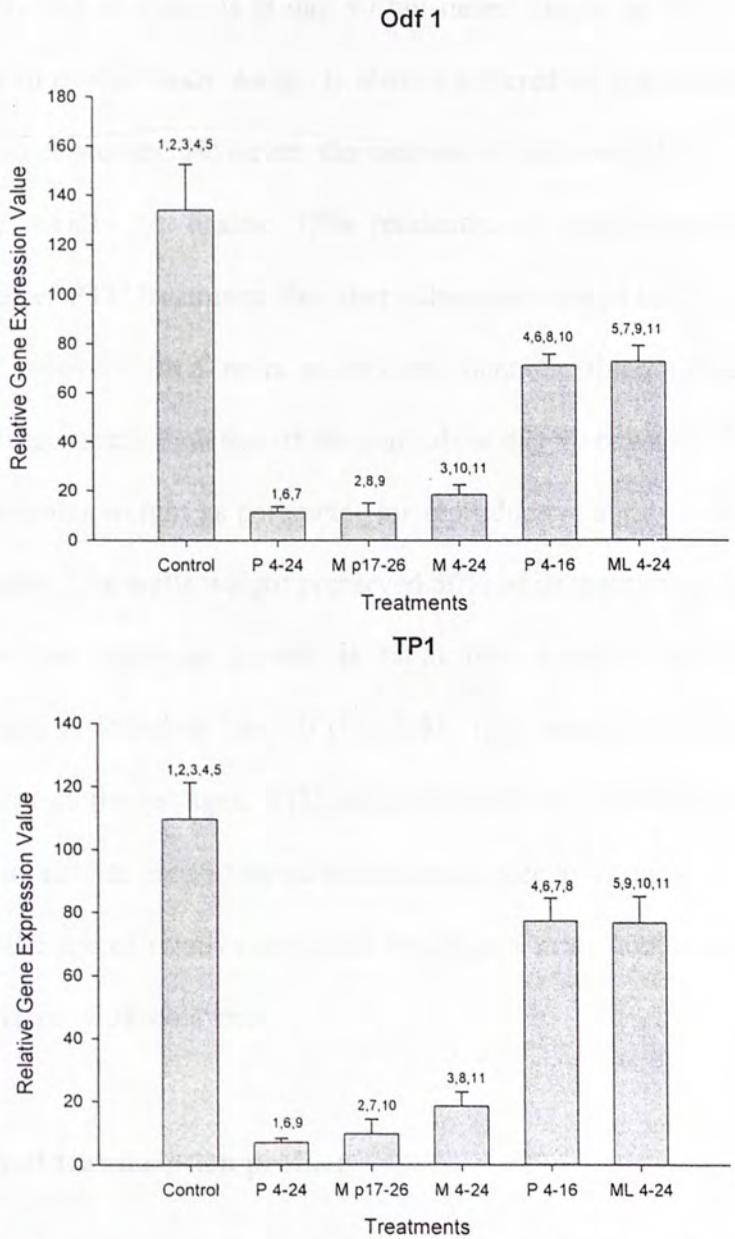


Fig 6.9 Transcriptions of Odf1 and TP1 genes under different hypothyroid regimens. Number pairs revealed statistical differences with $p<0.05$ by Dunn's test.

Discussion:

The pattern of body weight suppression in this neonatal hypothyroid treatment is similar to previous reports using same regimen. A rapid catch-up growth is seen soon after the cessation of rats under PTU treatment, but their body growth eventually paralleled with that of controls at day 90 but never caught up (Fig 1.1). A constant 20% decrease in overall body weight is always suffered by hypothyroidism, which is similar with other studies. However, the increase in testis weight is not parallel with that of body weight. A drastic 75% reduction of testis was seen during and immediately after PTU treatment. But after subsequent return back to euthyroid state, testis weight recovers in a more prominent manner, which eventually having a significantly larger testis than that of the controls at day 90 onwards (Fig 1.2). The use of relative testicular weight as parameter for reproductive ability revealed even more interesting figure. The testis weight preserved 50% of its mass immediately after PTU treatment, but the catch-up growth is rapid that a significantly bigger relative testicular weight is found at Day 50 (Fig 1.3). This increase seems becoming even more prominent as the rat ages. PTU administration affects the growth of rats in an overall ratio, as similar proportion of reductions in size and weight were seen in other organs. Thus the use of relative testicular weight is a more comprehensive parameter to reflect the fertility of male rats.

Five patterns of transcription profile:

Using neonatal hypothyroidism as a model of studying the transcription profile during testicular hypertrophy, I can identify and characterize my results into 5

different groups according to their temporal expression from puberty to matured adults:

1st pattern:

Transcription of this family of genes is almost undetectable before Day 24 for both control and hypothyroid groups. Starting from Day 30, expression of these genes within control group gradually increases until Day 50, when the transcription reaches the peak and plateau off at later ages. But in hypothyroid group, a lag of about 10-day in expression trend is observed. The transcription of these genes starts around Day 40 in PTU-treated rats, and continues to increase until Day 60 and reaches plateau. Thus a significant down-regulation of these genes was observed around Day 30 to Day 40 in the treated pups. Examples of genes that fall into this group are ADAM2, 3, 5, 18, 30, 32, PRSS21, Clgn, Cystain C and the marker of meiotic cells Lactate Dehydrogenase 3 C-chain (LDHc).

2nd pattern:

The expression pattern is similar with that of first pattern, with only a ten-day delay shift in the entire profile. Transcription of this family of gene is very low at/before Day 30. At post partum Day 40, expression started to rise and rapidly plateau of at Day 50. Hypothyroid group still suffered from that 10-day lag, with expression appeared as late as Day 50. Significant down-regulation was observed between Day 40 to Day 50 in PTU-treated rats. Example of genes that belongs to this category is ADAM4, ADAM 6, ACE, Nep, Cystatin 8, Bikunin and all post-meiotic spermatids markers like transition proteins (TP1 and 2) and protamine 2 (Prm2).

3rd pattern:

No significant age, treatment or interaction variation was seen after the two-way ANOVA analysis. Expression of these genes is not age-dependent. Neonatal hypothyroidism seems not suffer any lag in overall expression trend, thus no significant down-regulation was examined in all age groups after pair-wise comparisons. Genes like ADAM10 and ADAM17, uPA, α_2MG, β_2MG, TIMP2, Nedd4a and most cathepsins belong to this group.

4th pattern:

This group of genes is already highly expressed in testis as soon as Day 24, and rapidly peaked at Day 30 of the pre-pubertal stage. Expression at later stages remains constant. Hypothyroidism also suffers a 10-day lag within this family. Expression is extremely low before Day 30, but quickly climbed back to normal at Day 40. PCI, Cystatin SC and TE-1, Dncic 1 and MGP have this form of expression profile.

5th pattern:

Transcription of these genes was maximum before puberty, but suffers from a gradual decrease of expression when the rats age. This age-dependent down regulation is continuous until Day 90, when minimal is found. ADAM15, ADAMTS-1/4/5, Syndecan-4, tPA, TRα1, Claudin-1 and Cdh-8 etc have this expression profile.

This data of using neonatal hypothyroidism as model of studying fertility shares extreme coherence with a recent large scale micro-array expression profiling analysis[84]. In that experiment, four particular types of testicular cells: Sertoli cells, spermatogonia, pachytene spermatocytes and early spermatids were isolated with their expression of 11,955 chosen loci was investigated. Among the 1268 loci that display differential expression, they were organized into four main clusters according to their dominant expression localization: Somatic, mitotic, meiotic and post-meiotic respectively. Interestingly, all investigated genes that were clustered into same classification have identical expression trends that we have described. For example, ADAM2, ADAM3, and ADAM5, which suffered from down-regulation between Day 30 to Day 40, were all clustered as "MEIOTIC", while genes like ADAM 4 and ADAM 6, which shown similar down-regulation between Day 40 to Day 50, were all clustered as "POST-MEIOTIC". Most genes that have neither shown age nor hypothyroid dependencies in our experiments were clustered as "SOMATIC". This important finding may help us to guess the predominant location of expression for some other un-clustered genes, if this hypothesis of linkage is a real one.

Genes With known clustering information:

Meiotic	Post-Meiotic	Mitotic	Somatic
ADAM 2, 3, 5	ADAM4, 6, PACAP38,	GATA-4, Id-2	ADAM10, 17
Pkiβ,	Spam, Sp10, KRP2	TIMP-1, -3	α_2MG, β_2MG
Sert-1,	Prm2, TP1, TP2	Col3α1	Testin, Nedd4a
LDHc	Cystatin 8, Pcsk4	MGP	TIMP-2

Table 2: Summary of studied genes that with identified clustering information. Data are kindly provided by Dr. Ulrich Schlecht of Biozentrum, and Swiss Institute of Bioinformatics, Basel, Switzerland. Please refer to GermOnline at *http://www.biozentrum.unibas.ch/personal/primig/rat_spermatogenesis/* for most updated information in related subject.

Suggestion on the role of "MEIOTIC" proteases and inhibitors

This classification of proteases and protease inhibitors may also shed light on explaining their role of matrix remodelling during each stage of spermatogenesis. The establishment of tight junction is completed around Day 15 in rats[85], thus here I assume that the start of normal spermatogenesis at this age. Although the appearance of spermatocytes can be as early as Day 12, these cells will be eliminated without the protection of Blood-Testis Barrier (BTB). Undifferentiated type B spermatogonia normally take two weeks to become the first member of primary spermatocytes, the leptotene cells. During this stage, leptotene cells were arrested to wait for the transversion across the intermediate compartment of Sertoli cells. This implicates that before postpartum Day 30, leptotene cells need to secrete proteins that requires for continuous disassembly and assembly of tight junction protein. Notice that the expression of "MEIOTIC" proteases and protease inhibitors appears around Day 30, which evoke that "MEIOTIC" proteases maybe required for the digestion of tight junction protein, while "MEIOTIC" protease inhibitors are essential to maintain the integrity of blood-testis barrier at non-specific sites by suppressing the proteolysis.

Cell type		Postnatal day of first appearance
Undifferentiated spermatogonia	A_s Spermatogonia	4-6
	A_{pr} Spermatogonia	4-6
	A_{al} Spermatogonia	4-6
Differentiating spermatogonia	A_{1-4} Spermatogonia	4-6
	In Spermatogonia	6
	B Spermatogonia	6
Meiosis I Primary Spermatocytes	Pre-Leptotene	9-12
	Leptotene	9-12
	Zygotene	15-18
	Pachytene	15-18
	Diplotene	23-26
Meiosis II	Secondary Spermatocytes	26
Post-meiotic Spermiognensis	Round Spermatids	26
	Condensing Spermatids	38
	Spermatozoa	45

Table 3: Timing for first time appearance of male germ cell line lineage in rats. Morphological data based on morphological study from Clermont and Perey[4] carried out five decades before and is one of the classical studies in the related field. Table modified from McCarrey[86].

Genes that has been classified as "Meiotic" in our study are ADAM 2, 3, 5, Proprotein convertase subtilisin/ kexin type 4 (Pcsk 4), Sertoli cell protein 1 (Sert-1) and Protein kinase inhibitor beta (Pki β). Peaked expression of these genes in meiotic spermatocytes suggested that they are critical candidates in monitoring the process of meiosis. These genes may be involved in the cytoplasmic reformation from diploid spermatids to haploid spermatids. Trans-version across the tight junction also take place before maturation of spermatocytes, thus meiotic genes may responsible for maintaining the integrity of Sertoli cell barrier through refining the synthesis of proteases and its inhibitors.

Suggestion on the role of "POST-MEIOTIC" proteases and inhibitors:

If this hypothesis is true, I can continue this story as following: assuming successful spermatogenesis starts after the establishment of BTB at Day 15. After synthesizing "MEIOTIC" proteases and protease inhibitors, spermatocytes goes on differentiation to become matured spermatids, which normally takes around 14 more days. Therefore around postpartum Day 40, matured spermatids are developed and readily to be released from Sertoli cells. In order to hold the developed sperm avoiding the premature release, substances like protease inhibitor may necessary for reinforcing the apical ectoplasmic specialization. After completion of maturation, spermatids secrete another type of substances that digest the adhesive molecules for spermiation. Again, the expressions of "POST-MEIOTIC" proteases were not initiated before Day 40, which proposed that "POST-MEIOTIC" proteases may be responsible for the digestion of these Sertoli-germ cell junction complexes just before spermiation. Also, these genes may also be a component in the functional aspect of matured sperm, for example acrosomal proteins that facilitate the fertilization during sperm-egg membrane fusion or embryo implantation.

Genes that were catalogued as "Post-meiotic" are ADAM 4, 6, TP1, TP2 and Prm2, Sperm Protein 10 (Sp10, which is also called ACRV1, acrosomal vesicle protein 1), Sperm adhesion molecule (Spam), and Outer dense fiber of sperm tail 1 (Odf1). Notice that most of these genes are markers of mature spermatids that assist fertilization through sperm-egg binding or translocation, thus "Post-meiotic" proteases should be responsible for spermiation or fertilization process, while protease inhibitors should be in charge of inhibiting the activation of acrosomal proteases or reinforcing the ectoplasmic specialization.

Explanations on "SOMATIC" genes:

Previous studies proposed that testicular hypertrophy induced by neonatal hypothyroidism is due to the increased number of Sertoli cells; potentially increase the available number of sperm that can be supported. Unlike FSH, thyroid hormone cannot directly stimulate the mitosis of both Sertoli cells and germ cells, meaning that hypothyroidism can only slow down the maturation of Sertoli cells, thus prolonging the proliferative period for replenishment. From my data on "SOMATIC" gene transcription profiles, most of them showed no specific age or treatment dependencies. "Somatic" genes are referred to genes that are mainly expressed in Sertoli cells in testis, but also presents in other somatic tissue like brain and bone. Hence somatic genes are genes that are widely expressed, and sometimes noticeable amount of transcript is present even in spermatogenic germ cells. Due to the large diversity of expression in varies tissues; these genes should be responsible for some universal functions that are required by most cells, for example signal transduction or housekeeping. Their role on direct matrix remodelling is less emphasised. Genes that were grouped as "Somatic" are Cathepsin-L and B, tPA, ADAM 10 and 17, global protease inhibitor α_2MG and beta-2 Microglobulin (β_2MG), TIMP-2, cell junction proteins like testin, and Neural precursor cell expressed, developmentally down-regulated gene 4a (Nedd4a).

The development of Sertoli cells is entirely in the neonatal period, which is much earlier than that of our experimental design which begins at Day 24. Thus extending the temporal study starting from Day 4 may be helpful in understanding the participation of "SOMATIC" genes. It is known that neonatal treatment of PTU starting at Day 8 is unresponsive in increasing the testis mass and daily sperm

production in adult[87], which proposed that the critical period of thyroid hormones on Sertoli cells may be limited to a period before Day 8. So the transcription of "SOMATIC" genes should be studied at neonatal ages, otherwise the pattern will be similar with those in our experiment, which already plateau off for both control and hypothyroid at Day 24.

Explainations on "MITOTIC" genes:

This investigation over several "mitotic" genes failed to identify a specific clear-cut expression pattern as reported in previous mentioned model. For example, in the case of TIMP-1 and Matrix Gla Protein (MGP), they show down-regulation during early stages of PTU-treated pups and recover to normal levels after Day 40. But from my preliminary screening data over families of small GTPases like RhoA, cdc42 and Rhes and transcription factors GATA4, gene expressions seem to show some trends of up-regulation during the pubertal stage of hypothyroid rats. Moreover, genes like Id-2 and GATA-4 have clear trend of age-dependent down-regulation during the development. Therefore genes that are classified as "mitotic" may not need to share a homogenous expression profile within the group. It is known that mitosis in spermatogonia can be either "Proliferative" (for self-renewal) or "Differentating" (for gamete production), thus identification of genes that regulate these two individual stages may requires more refined isolation method. Histological isolation of proliferative and differentiating spermatogonia before clustering study can give clues in suggesting the role of these genes during the mitosis.

Explanations on the un-clustered pattern:

This type of genes showed an exceptional high transcription at the pre-pubertal age of Day 24. Although the expression is peaked at early ages, neonatal hypothyroid state still affects the expression of these genes before Day 30. Many members within this family was investigated but not being clustered, meaning that they do not display specific differential expression over any particular testicular cell types. These genes may participate in a more general or common purposes that shares similarities between Sertoli cells and germ cells, for example activation of cytokines or receptors, or being a candidate in universal signal transduction or cytoskeletal reformation during the development. These genes also suffered a severe down-regulation due to the hypothyroid treatment from day 24 to 30. If the 10-day lag is still applicable, the critical functional period of these genes under normal condition should be around Day 15, which is the terminal period of which Sertoli cells cease dividing. These genes may be the true contributors in deferring the cessation of Sertoli cells' proliferative period, and genes involved maybe expressed in multiple types of testicular cells but not only in Sertoli cells.

Explanations on the age dependent down-regulation group:

Most of these genes encode secretory product that are produced by Sertoli cells. Significant age-dependent decrease of overall expression suggested that these genes are responsible for testicular development before puberty, i.e. Sertoli cell maturation and tight junction formation. Decreased transcriptions over the age suggest that these candidates may not be the main participants in spermatogenesis. Another reason for this down-regulation may be due to changes in the proportion of testicular cells.

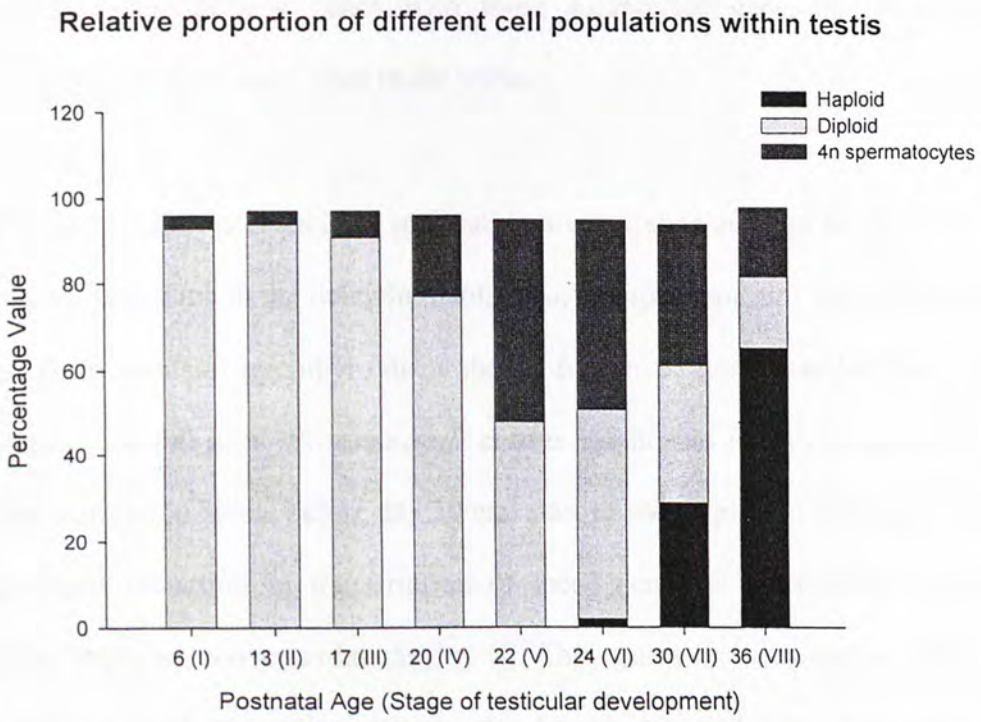


Fig 7. Relative proportions of n, 2n and 4n cells within the testis. Appearance of haploid spermatids can be as early as postnatal day 24, but significant proportion is achieved until day 30, and becoming the major constitutional cell type in testis afterwards. Data are obtained from Malkov *et al.*, 1998.[88]

Before puberty, major constitute of testis are diploid spermatogonia and somatic cells as shown in Fig 7. When the rat grows up, spermatogenesis starts over and increases the overall proportion of spermatocytes. Eventually in the adults, haploid spermatocytes and spermatids contribute to most of the cell population in testis, making the transcription levels in Sertoli cell to the whole testis relatively lower.

Proposed clustering of genes according to their transcription profile:

Clustering of some target genes in this study has already been known, but large proportion of them yet needs to be identified. By comparing the unique and distinct transcription profiles among different target genes, I am able to correlate my findings

on certain uncharacterized genes with those established genes to predict their predominant gene expression sites in the testis.

It is known that post-meiotic spermatids are not appeared before day 30 under normal condition. Due to the delay in maturation, the appearance of spermatids in rats suffered from neonatal hypothyroidism should further be postponed for two weeks. Thus genes that fall into "Post-meiotic" cluster should have two characteristics: 1. Very low expression levels before day 30 and start to rise to plateau levels afterwards. 2. Significant reduction in transcription of these genes in PTU-treated rats with expression starts to recover as late as day 60. Thus genes like calpastatin, KRP2 and others share similar expression pattern with known "Post-Meiotic" candidates like ADAM4 and ADAM6 should belong to the same cluster. This clustering idea can be validated by some well-known markers of specific spermatogenic cells. In this case, the TPs and Prms which are specific markers of post-meiotic spermatids also expressed in a similar pattern.

Using similar approach, identification of genes belonging to meiotic cluster is carried out. When considering the markers of diploid spermatocytes, several characteristics of meiotic expression profile can be drawn. Very similar with those in "Post-meiotic" cluster, gene expressions of "meiotic" genes are extremely scarce due to the low abundance of spermatocytes before puberty. Soon after the initiation of gametogenesis, the proportion of spermatocytes begins to rise at postnatal day 30, which starts the appearance of "meiotic gene" transcription. Under neonatal hypothyroidism, a ten-day delay in maturation of Sertoli cell is resulted, making the appearances of these meiotic genes postponed to day 40 when a significant down regulation resulted. At day 50, the relative testicular weights in PTU-treated litters

have already overshot that of the normal, reflecting that the testicular environment (in terms of proportions of gem cell in testicular population) should be comparable, thus no differences in overall transcription is foreseen. It should be reminded that even the transcription profiles of "Meiotic" and "Post-Meiotic" cluster are highly symmetrical where the first time appearance in hypothyroid rats is the only discriminating factor, the identification of these two individual clusters must be clear-cut without confusing. Spermatids would not synthesize "meiotic" mRNA; the appearances of meiotic proteins are the translation products of internal transcript store. Yet the expression of "meiotic" genes is always appeared in spermatids also, the regulatory mechanism of transcription in these two clusters is completely different. Meiotic spermatocytes are mainly diploid while post-meiotic spermatids are all haploid. Unique critical period of expression and pre-dominant localization suggested that the putative functions of the two gene cluster can be overlapping but distinct from each other.

Proposing cluster of genes by current profiling	
Meiotic	**Post-Meiotic**
ADAM 18,30,32	ADAMTS-16
Clgn, PRSS21,	MT3-MMP, tACE,
Bikunin, Spink2	Zfp37, PACAP38,
Cystatin C	Nep , TIMP-4
	Calpastatin, Act, Eppin

Table 4: Suggested spermatogenic clustering of some uncharacterized genes in this study. The clustering idea is based on the unique expression profiling under different developmental stages as described. Owning to some limitation, this clustering idea is only applicable to identification of spermatogenic (meiotic and post-meiotic) candidates.

The prediction of "Mitotic" and "Somatic clusters" is probably impossible in the current study. As discussed before, mitotic germ cells are not of homogenous in origin.

Different types of mitotic spermatogonia i.e. proliferative or differentiating strains should have different genetic requirements, thus no unique profile feature can be clearly drawn. Moreover, peritubular and interstitial cells may also express mitosis-related genes for cell replenishment, making the identification of mitotic cluster even more difficult. In my study, mitotic gene cluster refers to genes that preferentially transcribed in mitogenic spermatogonia only, thus histological isolation before expression studied is required to outline their developmental profile.

Sertoli cells are the main constitute of "somatic" cluster in the micro-array study. However, Leydig cell and other peritubular somatic cells are also transcriptionally active. It is therefore better not to define any unique expression profile for "Somatic" cluster in this experiment. In this experimental model, the mRNA is coming from the whole testis and thus is of multiple-cell origin. Relative proportion of Sertoli cells is actually decreasing with age, thus the transcription profile of Sertoli Cell-predominant genes should actually decreasing though their expression within cell should not be affected. As discussed before, critical period of Sertoli cell proliferation is entirely before the puberty. The expression of Sertoli cell specific genes may not be altered within day 24 to 90 in this study. Using these two characteristics (age dependent down regulation and no hypothyroid dependency), I suggest that genes like ADAM15, tPA, Cystatin SC, Syndecan-4, TRα1, Calpain I would be preferentially expressed in Sertoli cell mainly.

"SOMATIC" genes expressions are neither affected by age nor treatment. Since these candidates are widely expressed in both Sertoli and germ cells, the temporal expression profiles of the whole testicular tissues can not be used to identify whether they are Sertoli or germ cell specific. The constant and stable expression of these

genes is not simply the summation of age-related up regulated spermatogenic portion and age-related down-regulated Sertoli cell portion. The transcription profiles of somatic genes are heavily influenced by their preference on site of expression and the relative proportion of cell population during that particular age. For simplicity, here I suggest genes that are constantly expressed without age or treatment dependency as "Somatic" cluster; meaning their site of expression is diverse and complicated.

Proposing cluster of genes by current profiling	
Sertoli cell predominant	**Somatic (Mixed originate)**
ADAM15, ADAMTS-1, -4, -5	cdc42, Ras GRP-1, Rhes, Rho A,
tPA, Cystatin SC, Claudin-1,	uPA, PAI-1, sACE
Cadherin-8, Syndecan-4, TRα1,	Cathepsin L, B, C, MMP-9,
Calpain I, Nexin-1, PACAPr	Axin, Sertolin, Occludin, Claudin-11,
	E-/N-Cadherin, Cadherin-11, Cx43

Table 5: Suggested non-spermatogenic clustering of some uncharacterized genes in this study. Profiling of these two gene clusters is much more complicated and far less reliable than that of the two spermatogenic clusters using the current model. This clustering idea requires further immuno-histological or even laser assisted microdissection RT-PCR investigations for confirmation of their site of expression.

Now I want to take ACE as an example to describe my idea on gene profiling. The ACE gene encodes both a somatic and testicular isozymes. The former one (sACE) is widely expressed in many somatic cells, including endothelial and Leydig cells in testis. But the latter one (also named as testicular or germinal ACE, tACE) is a testis-specific form, which is predominantly expressed in spermatids and spermatozoa[89]. sACE consists of both N- and C-domain, while tACE is a splice variant of sACE with deletion of N- that cleaved in germ cells. Although N-domain is responsible for ecto-domain shedding and catalytic function[90], infertility is resulted in knockout mice deficient in tACE but not in sACE[52].

Here I report the transcription of sACE (between somatic-specific region and shared sequence), tACE (the region between the testicular-specific region and the shared sequence) and ACE, which is a common sequence at the 3' end of the whole gene.

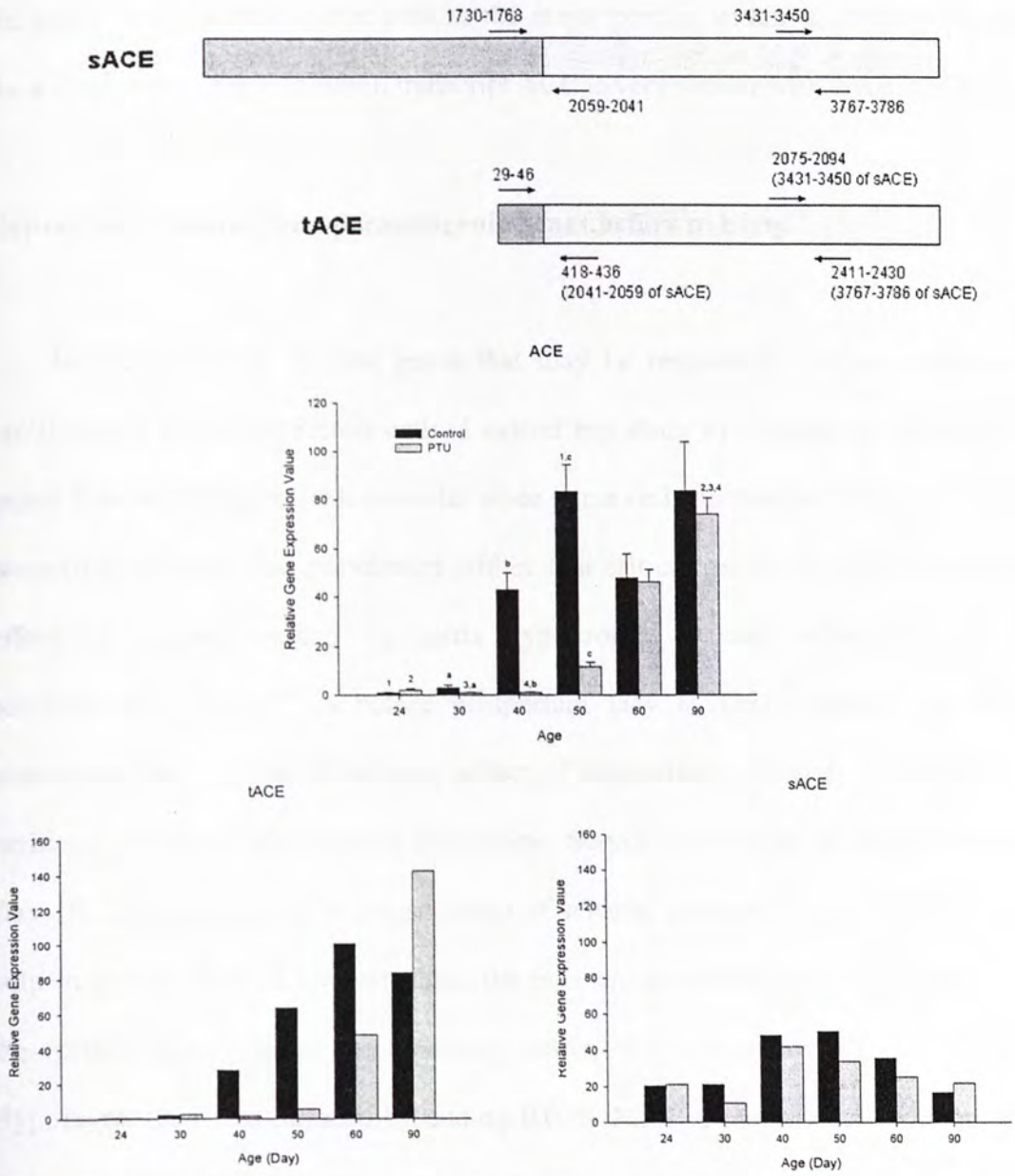


Fig 8. Primers design of ACE, sACE and tACE and their respective transcription profiles. tACE is an truncated form of sACE, but a specific sequence is also present in testicular form. Based on these primer designs, transcription profiles of the three sequences are shown above. Both ACE and tACE share a similar expression with the characteristic post-meiotic pattern, while expression of sACE seems constant throughout age and treatment.

Consistent with my prediction, spermatid-specific tACE shows the characteristic "post-meiotic" profile as previously described. The somatic isozyme sACE that widely expressed in multiple cell types also presents the typical "somatic" profile without age or hypothyroid dependency. Since tACE is the predominant isoform in the testis, with spermatogenic cells as the major portion within testicular population, the expression of their common transcript ACE is very similar with that of tACE.

Expression of some non-spermatogenic genes before puberty

In order to look for the genes that may be responsible for the extension of proliferation period of Sertoli cells, I extend my study by focusing to candidates of genes that are not germ cell specific, since germ cells do not contribute for a major proportion of testicular population within this critical period. It is known that the effect of hypothyroidism on testis hypertrophy is only effective when the administration of PTU is before postpartum Day 8. This suggests that thyroid hormones may take its stimulatory effect of maturation of Sertoli cell at the early perinatal period. Under normal conditions, Sertoli cells cease proliferation around Day 16. Temporal study on transcription of selected genes at Day 8, 16 and 24 may help in giving clues of understanding the mechanism of how hypothyroidism extend the proliferation period by slowing down the maturation of Sertoli cells. Hypothyroidism was induced by adding 0.05% MMI in mother rats' drinking water from prenatal day 16 instead, and treatment continues until pups were scarified. (Result on this part of experiment is attached in the Figure 4 as follows). LDHc, marker of meiotic germ cells, is also studied for comparison.

Again, no unified expression pattern can be drawn from this experiment. Result of LDHc validated that meiotic germ cells are present in normal rats as soon as day 24, but still absent in hypothyroid rats. All of the studied genes have not been affected by hypothyroid treatment before Day 8, suggesting that the action of thyroid hormone on transcription modulation is a gradual but not an immediate process. At Day 16, when the Sertoli cells start to cease proliferation, the expressions of cystatins, Claudin-11 and Clgn start to rise, but the expression in PTU-treated pups remains lower at their basal levels. This down-regulation of cystatins due to hypothyroidism is extended up to Day 30. Down-regulation of Claudin-11 further validates that the delay and impairment of tight junctions suffered from neonatal hypothyroidism. Lowered expression of inhibitory cystatins may be a factor in lengthening the maturation of Sertoli cells, hence providing a greater capacity for cell division. In contrast, the uncharacterized ADAMTS-16 and TIMP-4 is highly up regulated during the postnatal period with a drastic drop before entering the puberty. Since the substrate for this putative secretory protease ADAMTS-16 is still unknown, the paradoxically high expression of ADAMTS-16 together with TIMP-4 genes at neonatal period may responsible for the perinatal development, for example sex determination.

Even though the molecular mechanism of testicular hypertrophy in neonatal hypothyroid rat is still unknown, it was proposed that cell-cycle regulators are possible candidates for prolonged Sertoli cell proliferation. Two cyclin-dependent kinase inhibitors (CDKI) $p21^{Cip1}$ and $p27^{Kip1}$, which inhibit the action of cyclin-cdk complex to progress through G1 checkpoint, are shown to be involved in Sertoli cell proliferation[91]. Knockout mice lacking $p21^{Cip1}$ and $p27^{Kip1}$ have increased Sertoli cell numbers by 100% and 50% respectively[92].

Neonatal hypothyroidism induced testicular hypertrophy as a model for studying reproductive physiology

The use of neonatal hypothyroidism as model to study male reproductive physiology is quite well-established. Morphological data supported the role of thyroid hormone on Sertoli cell maturation. The formation of tubular lumen, which is a marker of seminiferous tubule development and Sertoli cell differentiation, is not seen even until day 30 under PTU-treatment, where its appearance should be as soon as day 20 normally[93]. Also, there is an increase in degeneration of spermatocytes and spermatids in pups suffered from hypothyroidism, suggesting that the establishment of tight junctions or other testicular environmental factors is not yet completed before day 30 under this neonatal hypothyroid treatment. It is now compromised that the increased size of testis is due to prolonged proliferation of Sertoli cell. In our experiment, the delay of differentiation (in terms of mRNA transcription) is at least ten days or more. Although the actual delay in maturation is slightly different among different studies, it was evidenced that mitogenically active Sertoli cells is still appeared in postnatal day 25 under PTU treatment[94]. This may imply that neonatal hypothyroidism provides a good model in studying the development and differentiation of testis around the puberty.

The response of gene expressions to T_4 replacement on hypothyroid rats is quite consistent in this study. In my second set of experiment (4.1 to 4.11), all genes with lowered transcription levels in the neonatal hypothyroid rats are able to restore to normal levels at certain extend upon replacement. A single dose of T_4 at day 35 restores the transcription of hypothyroid rats back to normal at day 40. This effect of replacement therapy is however not long lasting. An injection of T_4 at day 35 is not

able to restore the transcription of several target genes back to normal at day 50. It is because the dosage of T_4 used in this experiment is relative low and the biological half life of T_4 in rats is only around six days. The results of this replacement therapy study show us whether the examined target genes are thyroid hormone responsive or not.

In order to rule out the possibility of toxic effect due to the PTU treatment, the third set of experiment was carried out. From the results of this experiment using different experimental hypothyroid induction regimens, I try to validate whether alternation of gene transcription in the testis depends on different levels of experimental hypothyroidism (Results summarized in Fig 6.1 to 6.9). At Day 50, when most of the post-meiotic genes were still being down-regulated in the neonatal hypothyroid rats, shortening of the hypothyroid period reduces the responsiveness of certain target genes, though a significant difference is still present when comparing with that of controls. Comparing relative testicular weights within various treatment groups, the administration of PTU from day 4 to 24 is indifferent with same treatment that ends at day 16. This suggests that the induction of testicular hypertrophy in the neonatal hypothyroid rats may be earlier than day 16. When the effect of a less potent goitrogen MMI at a relative lower concentration (0.01%) was examined, not much difference can be found between the relative testicular weights between the 0.1% PTU and 0.01% MMI treatment groups. This implies that the degree of hypothyroid need not be a strong one to induce significant testicular hypertrophy. Same conclusion can be drawn in case of using MMI at the dosage of 0.0125%. Although the increase in relative testicular weight is quite stable among different treatments, expression of genes does not change in a completely symmetric way. In this experiment, all the five treatments can induce a significant increase of relative testis weight at day 50, but the

transcription of target genes under these cases is quite paradoxical. In most of the post-meiotic genes, no significant changes are found in groups that have milder hypothyroid treatment, but a drastic down-regulation is still present for pups that were given 0.1% PTU, 0.01% MMI or prolonged 0.01% MMI *ad libitum*. Why these high deviations in target gene transcriptions under different treatment regimen induce the similar levels of testicular hypertrophy is still not known. When I look into the transcriptions of mitotic or meiotic genes, no differences can be identified under different treatment regimens. This may suggest that post-meiotic genes may not be so critical for the development of testicular hypertrophy in this animal model.

Different components of spermatogenesis:

The development and assembly of sperms is a very complex biological event. Spherical diploid spermatogonia undergo multi-mitosis and eventually transform to bigger spermatocytes with genetic materials halved. Finally the haploid spermatids are remodelled into a characteristic shape with intense cytoplasmic reduction and flapping tail. In order to identify candidates that participate in this complex spermatogenesis, I emphasize on genes that are specifically or primarily expressed in testis only. Meiosis is solely restricted in spermatogenic cells, during which chromatin condensation, matching and separation are important landmarks. Hence nuclear division related proteins that are specifically expressed in testis only are though to be responsible for assisting meiosis. To drive the strong transcription preference over different type of cells, spermatogenic cells bear their own system of transcription control. Following the supervision from specific transcription factors, signal transduction molecules are essential for the subsequent protein trafficking and activation. Since remodelling of testicular microenvironment is the main concern,

different families of proteases and protease inhibitors, with their potential target: Junction and Matrix proteins were investigated. Developing spermatogenetic cells need to transverse across the seminiferous epithelium, thus a cytoplasmic motor that ensure the polarity of migration is required. In this section, I discuss the role of meiosis-related genes, transcription factors, signal transduction molecules, proteases and their inhibitors, junctional and matrix molecules together with motor proteins for their involvements in different aspects of male gametogenesis.

I. Roles of nuclear and chromatin related genes in assisting meiosis:

Meiosis is a prolonged nuclear division that allows sufficient time for a single diploid germ cell to double its DNA content, than halved twice to form 4 identical haploid. Pre-leptotene spermatocytes first synthesize DNA, while chromosome uncoiled and naked as separate chromatins. The homologous chromatins pair by forming synaptonemal complex in zygotene cell. After the genetic recombination of crossing over, chromatins start to separate and coiled with histones again to become chromosomes. Here I report the expression profile of Sycp1 (Fig 2.34), the major component of Synaptonemal complex protein, where its expression can be found in both meiotic spermatocytes (predominant) and post-meiotic spermatids (abundant). Expression of Sycp1 is already relative high at Day 24, when spermatid is still not present. This means that gene transcriptions at Day 24 are mainly taken place in spermatocytes or in somatic cells. This is followed by the appearance of round spermatids at day 30. High expression of various target genes after Day 30 is contributed by both meiotic and post-meiotic cell types. Expression profiling of Sycp1 is to demonstrate the pattern of transcription of genes that co-expressed in more than one type of testicular cells. Another family of chromatin-related genes are the TPs and

Prms. During the spermiogenesis, spermatid has its own system of chromosome condensation that is distinctive from normal somatic histones. Within these post-meiotic germ cells, histones were replaced by another small basic TPs and Prms for DNA binding and coiling[95]. Unlike synaptonemal complex proteins, all TPs and Prms are only expressed in signal cell types, making them an ideal marker for identification and isolation. These genes that have unique expression preferences show a completely different pattern with the previous one. Take TP1 and TP2 for examples, since their expressions are solely on spermatids[96], no transcription could be found until Day 40, while plateau expression afterwards is taken place in post-meiotic cells.

II. Roles of specific Transcription Regulators in assisting gene selection:

Because of the uniqueness of gametogenesis, gene expressions in meiotic spermatocytes are largely different with somatic cells, especially the chauvinist genes. These genes are transcribed only in spermatogenesis cells with a defined developmental dependency[97]. Chauvinist genes may be responsible for carrying out machineries that are independent from normal somatic cells, for example sperm assembly, meiosis or specialized junction architecture. In order to limit and select gene expression that can be utilized by specific cell types, testis has their distinctive transcriptional control mechanism. This specialized selection, either by expressing testis-specific genes or splicing of specific isoforms, is thought to be an evolutionary advance that can provide a more suitable microenvironment for reproductive gamete production which other somatic tissue absent. One of the most established testis specific transcription regulation is the cAMP-response element modulators (CREM). CREM encodes a bZIP protein that binds to cAMP-response element in the promoter

region. CREM consists of several splicing variants, surprising that most splice isoforms are repressive due to the removal of the activation exon. CREM transcription machinery can thus be regulated by its splicing process or upon activation by phosphorylation of other protein kinases, or expression of other proteins like cAMP-response binding protein (CREB) and Activator of cAMP-responsive element modulator in Testis in testis (Act). Apart from CREMs, the GATA family is also a marker transcription factor in the testis. Especially GATA4 is shown to be regulated by different endocrine factors like testosterone and Sertoli cell paracrines or autocrines in the testis. Yet another transcriptional regulators that also influenced by endocrine system is the **Inhibition of Differentiation proteins** (Ids), which suppress transcription of differentiation related genes by forming dimmers against bHLH motifs in the TATA-box.

Preliminary screening data of transcription modulators are summarized in Fig 3.1 of the Appendix. Diploid spermatogonia, haploid spermatocytes and spermatids together with somatic testicular cells are all transcriptionally active, thus at least 3 kinds of control mechanisms can be foreseen. GATA-4 [98] and Id-2 [99] are predominantly expressed in spermatogonia and showing the typical "mitotic" pattern, while Act[100] and Zfp37[101] which are mainly expressed on spermatids also revealed the characteristic "post-meiotic" profile. Expression of CREMδ[102] is thought to be more universal, multiple origin of expression is suggested. Pkiβ[103] is also a key regulator of transcription in germ cells together with CREM showed the typical "meiotic" profile.

Ⅲ. Role of Signal Transduction molecules for protein translation and activation:

For proper expression and functioning of proteins, signal transduction cascades are essential for carrying a great variety of purposes, for example post-translational structural modification, functional regulatory machineries through phosphorylation or adenylation, protein trafficking and intracellular communications. Unlike the specialized transcription regulations previously mentioned, signalling molecules are always not cell-specific, and is universally appeared in other somatic cells. **Protein kinases** like PKA and PKC; cAMP, G-proteins and small GTPases may be liable for the regulation of junction dynamics[104], since some major junction proteins like occludins, cadherins, catenins and integrins[10] have putative phosphorylation sites. It was also demonstrated that a transient increase in cAMP and/or cGMP was observed during the assembly of tight junction, but return to basal level once the construction completed.

Small GTPases are involved in various cellular functions, for example regulation of gene expression, adhesion, cell cycle progression and apoptosis[105], which are all important for development of the testis and spermatogenesis. In my investigation over the Rho subfamily of cdc42 and RhoA; Ras GRP-1 and Rhes, the expression of these small GTPase seems not dependent on age and treatment (Fig 3.2). Sertoli cells are their predominant expression site, while profound abundance was also detected in germ cells. Constantly high expression throughout life and without treatment dependency may implicate that these candidates are of universal housekeeping functions and less related in functional spermatogenesis. Neurotrophin-3 (NT3), which shown to involve in testis morphogenesis[106], has the unique transcription profile that similar to Cadherin-8 and TRα1.

Implementation of messengers or functional proteins always requires interaction of other enzymes for their activation. Proprotein convertases are good example of this. Proprotein convertase subtilisin/kexin type 4 (Pcsk4) is a putative substrate for pituitary adenylate cyclase-activating polypeptide (PACAP) activation[107]. PACAPs are growth factors that are critical in cell proliferation and differentiation[108]. Together with their specific receptors (PACAPr), these candidates can alter the signal transduction during spermatogenesis. Other kinds of putative substrates of proprotein convertase are the main concern in my study, the ADAMs. Most ADAMs contains a proprotein convertase cleavage site between their pro- and metalloprotease domains[109]. Cleaving of pro-domain is necessary for the proper trafficking across ER and its conformation folding. In the current study (data summarized in Fig 3.3), Pcsk4 and PACAP38 belong to meiotic and postmeiotic cluster, while PACAP receptor (PACAPr) should be Sertoli cell predominant by prediction. This may imply that germ cells may actively secrete enzymes and signals to the surrounding Sertoli cells for establishing spermatogenesis.

IV. Role of proteases and inhibitors for matrix and junctions dynamics

The high versatility of proteases and their endogenous inhibitors during spermatogenesis has been discussed previously according to their main expression sites. Apart from structural remodelling by proteolysis of ECM and junction components, proteases can activates cytokines to activate different signal transduction cascades. They can shed and activate proteins or ligand receptors to modulate membrane specificity and their response. Testicular proteases and their endogenous inhibitors can further be divided into 2 subclasses: somatic and spermatogenic:

The somatic proteases and inhibitors system in the testis:

ADAM10[110] and ADAM17[111] are well known candidates of intercellular signalling for their sheddase activity of Notch ligands and TNFα. Gelatinase B (MMP-9)[112], Cathepsin L[113], uPA[114] and calpain I have been reported to be involved in the matrix remodelling in the testis. Nexin-1[115] is another protease that inhibits other proteases like tPA, uPA and thymosins. (Transcription profiles of these genes are summarized in Fig 3.5 and 3.6 of the Appendix). All these are example of somatic proteases and inhibitors that presents in Sertoli cells.

In case of the PA system, time-dependent down-regulation of tPA was observed in both control and hypothyroid rats (Fig 2.18), while the expression of uPA seems to be unaffected (Fig 3.5). In this experiment, PA inhibitors like PCI (PAI-3) was highly expressed from pubertal to adult control rats (Fig 2.29), which may account for the continuous degeneration of PAs throughout the time. Hypothyroid rats benefits from a 10-day delay in the boosting of inhibitors expression. This delay may provide a longer period for the action of PAs for matrix remodelling or cytoskeleton modification that can assist in extending the proliferative period of Sertoli cells. Spermatogenesis is one of the continuous lifetime machinery in mammalian testis. Down-regulation of tPAs throughout age advancement implicates that PAs may not be the main contributors of spermatogenesis, but play more important role in the early development of testis between prenatal to pubertal periods. Similar correlation was observed in the **Calpain** and **Calpastatin** system. Calpain I, a calcium-dependent widely expressed cysteine protease, is down-regulated in the aging rats. Its endogenous specific inhibitor, calpastatin[116], also suffered a 10-day delay in its expression

profile under the neonatal PTU treatment even if it should be spermatids-originated. Thus evidences are provided that the critical period of somatic proteases and inhibitors that regulate the junction dynamics is limited to the pre-pubertal period that is critical for Sertoli cell proliferation. These genes may be responsible for the molecular maturation of Sertoli cells or premature assembly of blood-testis barrier. However, proteins encoded may not be the essential candidates in maintaining the microenvironment for spermatogenesis or sperm maturation.

Spermatogenic proteases and inhibitors system:

Most of the studied proteases (especially ADAMs) and inhibitors that predominantly expressed in developing germ cells have a completely different picture with that of the somatic PAs. In contrast, these genes were apparently undetectable before puberty with a drastic rise in their transcription was observed just before the sexual maturation. This kind of expression profile is symmetrical with the reproductive period of rats. Normal male rats would not be fully sexual mature until Day 50. Sperms isolated from pre-mature rats are not optimal for fertilization. High expression level of target genes maintained in the testis of mature male rats suggested that these genes are responsible for the modulation of spermatogenesis. Dislike the cases of somatic genes, both spermatogenic proteases and inhibitors were expressed at high levels. Excessive production of the two counteracting families suggested that both candidates are required to sustain the process of spermatogenesis. High expression of protease inhibitors is required for protection of unwanted proteolysis and upkeep the rigidity of Sertoli cell barrier, at the same time, expression of specific proteases are essential to

remove these obstacles for maturation.

Transcription of meiotic proteases and inhibitors are only confined in meiotic spermatocytes, thus their role should be related with processes like Sertoli-Germ cell interactions or meiosis-related machinery. In this experiment, expression of these meiotic genes reaches plateau at about day 40 in normal rats. Pups suffered from neonatal hypothyroidism have shown significant down regulation in their transcriptions with a ten-day delay in their expression profile. In case of the post-meiotic genes, expressions still maintain the plateau levels at day 40 in controls, while their transcriptions cannot been restored even until day 60. This means that the post-meiotic genes may even suffer up to a twenty-day delay in transcription under the neonatal treatment. Post-meiotic genes are supposed to be involved in process of spermiogensis and fertilization. A further delay of ten more days of their peak expression levels indicates that the neonatal hypothyroidism exert an even more extensive effect in spermiogenesis. Sperms from neonatal goitrogen treated euthyroid adult rats are completely normal in terms of morphology and their fertility. It can happen that thyroid hormones are also involved in machineries that related in cytoplasmic remodel of spermatids.

The expression of testicular proteases and their endogenous inhibitors are summarized according to their catalytic group: Serine, cysteine, aspartic and metalloprotease. In this experiment, plasminogen activators uPA and tPA, together with the uncharacterized PRSS21 are the only serine proteases being studied. Although widely expressed in many testicular cells, main expression site of PAs are Sertoli cells. Our finding in this study is that PA is down-regulated in advanced ages while the expression of uPA is not temporally affected. But when I look into their

specific serine protease inhibitors (Serpins), interesting finding showed that PAI-1 and PAI-3 (PCI) are also expressed in a constantly high level. Calpain I and the lysosomal cathepsin L and B are cysteine proteases and their expressions are also mainly on Sertoli cell. Most of them are assembled in pro-enzyme form, thus activation by other proteases may be needed. Current study indicated that cathepsins are all not affected by age or neonatal hypothyroid treatments and calpain I is also down-regulated in advanced age. Specific cysteine protease inhibitors, Cystatins, are extensively studied here. Although the expression profiles of Cystatin 8, C, SC and TE-1 is similar with those of spermatogenic members, high transcriptions are also detected at earlier ages in control pups, meaning that the expression site of cystatins are co-expressed in both somatic and spermatogenic cells.

The characterization of testicular aspartic proteases is not that established when compared with the other three groups. Cathesin D and ACE are the only candidates in this experiment. Cathepsin D is widely expressed in Spermatocytes, Sertoli and Leydig cells. Expression of the two ACE isozymes, somatic and testicular forms, is non-overlapping. No endogenous and specific inhibitor of aspartic proteases is identified. But global binding globulins like α2MG and β2MG can virtually inhibit the activities of all proteolytic enzymes. These macroglobulins are also widely expressed, with Sertoli cell as their main origin.

Metalloproteases and their inhibitors are the main focus in this study. All ADAMs and ADAMTSs, MMPs and the Nep belong to metalloproteases. Apparently most of these metalloproteases are spermatogenic origin when identified using the clustering idea. Most of them possess unique developmental profiles and are down regulated upon hypothyroidism before sexual maturation. The expression of the four

tissue inhibitor of metalloproteases (TIMPs) is however quite heterogeneous. TIMP-1 and -3 are mainly expressed in spermatogonia, TIMP-2 in Sertoli cells while TIMP-4 in spermatocytes and spermatids. The interaction of proteases and their endogenous inhibitors is still not very clear and substance identification would greatly help understanding their role on mammalian reproduction.

V. Role of matrix and junctional molecules for intercellular interactions

One of the limitations of proposing the role of these testicular proteases and their inhibitors is the lack of information on their substrate specificity. I try to correlate my experimental results on some of the putative substrates, especially the matrix and junction molecules that are essential to produce a unique microenvironment for gametogenesis. Apart from the structural and adhesive reinforcements, ECM can also serve as reservoir of cytokines and signalling molecules and serves as platform of signal transduction upon release by proteolysis.

Sertolin[117], an extracellular matrix protein, is a marker of cell-cell interactions that link with Sertoli cells. Testin[118] is one of the integral anchoring proteins that mark the intergrity of Sertoli-germ adhering junctions, while Axin[119] is the adaptor that assembly and reinforce their interactions. Occludin and Claudin; Catenin and Cadherins [120] as well as Cx-43 [121] are markers of occluding tight junction, Sertoli-germ adhering junction and gap junction respectively. Though their importance in junction assembly and dynamics were already well-established, no significant age-dependencies of investigated genes can be observed in this study (Transcription profiles of above genes are highlighted in Fig 3.7 and 3.8 of the Appendix). Role of these junctional proteins during sexual maturation and

spermatogenesis still needed to be addressed.

Collagens are one of the major constitutes that make up of the extra-cellular matrix. In our experiment of studying the developmental expression of Col3α1, a tread of age-dependent down-regulation like tPA and syndecan-4 is clearly shown. Another adhesive protein family that presents in ECM are the syndecans. Syndecans are proposed to carry out its adhesive function together with ADAMs family and integrins. Similar with that of Col3α1, a significant down-regulation of syndecan-4 is observed. When comparing with the expression profile with that of spermatogenic proteases, it is interesting to figure out that the transcriptions of these proteolytic enzymes are inversely proportion to that of the major components of ECMs. This may suggest that ADAMs present on spermatogenic cells are responsive in matrix remodelling during spermatogenesis through its interaction with adhesive ECM components.

Although similar age-dependent down-regulation can be seen with claudin-11 and cdh-8, most of the other junctional components were not affected. Considering the developmental profile of other junctional proteins, e.g. the tight junction protein occludin, anchoring junction protein N-/E-/P-Caherins, or gap junction protein Cx43, no significant trends of age or treatment dependency are evidenced. It may imply that all these gene candidates play an important role in the assembly of Sertoli cell junctions and maintenance of hardware that happens before and during spermatogenesis. RT-PCR data from other group using same hypothyroid treatment also revealed similar findings: Immunohistochemistry data revealed that these junctional proteins which used to be expressed onto plasma membrane surface in normal group were being captured within cytoplasm under hypothyroidism[122].

Although the overall expression in the whole testis is not changed, but increased proportion of non-functional cytoplasmic junction proteins would significantly affect the assembly of testis dynamics. These findings may suggest that the neonatal hypothyroidism affect these junction proteins not by alternating their transcription levels, but changing the localization or trafficking of these proteins. This explanation can further be proven by the same morphological study, since the assembly of seminiferous tubule lumen is also affected under hypothyroidism. Hence alternation of intracellular signalling or cytoplasmic motor proteins may be responsible for the change in localization of these junction components.

VI. Role of cytoplasmic motors in cellular movement

Several theories of how the developing spermatogenic cells transverse across the seminiferous epithelium have been proposed[10], but clearly cytoplasmic motors are required for the developing germ cells to squeeze through crypts of Sertoli cells continuously. Kinesins are known molecular motors that responsible for force generation during variety of mobile activities within cells. The **kinesin-related proteins (KRPs)** are though to be involved in spindle dynamics during meiosis in particular [123]. **Dynein intermediate chain 1 (Dncic1)** was proposed to be microtubule-based motor, in which the translocation of germ cells by ectoplasmic specializations is supposed to be relied on microtubule cytoskeleton[124]. By the investigation on the transcription profile of these motor proteins (Fig 3.4), it is clear that KRP2 and Dncic1 do not share similar functional responsibility. KRP2, which is known to be spermatid-specific, conveys motor machinery after transformation into haploid spermatid and Dncic1 should involve in the early stages of spermatogenesis.

Conclusions:

Proposed story of spermatogenesis – involvement of proteases and inhibitors

Proteases like tPA and ADAMTSs are expressed extensively to build up the matrix architecture for the maturation of Sertoli cell and tight junction formation before the puberty. After the establishment of BTB, the testis is ready for the process of spermatogenesis to start the production of male gametes. Spermatogonia start to divide and differentiate into primary spermatocytes within the basal compartment. To prepare for the meiosis into the haploid daughters, the leptotene spermatocytes start to synthesis "meiotic" protease inhibitors to protect from proteolysis and retain blood-testis integrity together with the endogenous somatic inhibitors secreted by Sertoli cells. When coverage of protection is completed, "meiotic" proteases from spermatocytes are activated at specific contact sites on Sertoli-germ cell junction, opening spaces for migration across the intermediate compartment. After the migration into adluminal compartment, the presence of "meiotic" and "somatic" inhibitors stops the proteolysis and thus closes the tight junction. While spermatocytes continue to differentiate into spermatids, specific transcriptional controls are liable to activate the expression of varies spermatogenic proteins for modulating the cytoplasmic transformation with the support from different signalling molecules. During this transition, "post-meiotic" inhibitors are also synthesized to reinforce the adhesive junctions and ectoplasmic specialization to avoid premature release. Until the spermatids are fully matured and ready to be released, "post-meiotic" proteases are secreted and activated to breakdown the trapping junctions for the process of spermiation, in addition, latent forms of these "post-meiotic" proteases can serve as

media for oocyte recognition and fusion. During the maturation of germ cells, Sertoli cells provide a fine-tuned environment by remodelling its shape and interacting junction. "Somatic" proteases are constantly synthesized for activation of cytokines and receptors that participate in signal transduction cascades to provide functional support over the spermatogenesis.

Future directions:

Many additional efforts are required to validate the role of ADAMs on the development of testis and process of gametogenesis. The difficulty to substantiate this concept is lack of information on substrates that ADAM and ADAMTS are dealing with. Most efforts were focused onto the two universally expressed somatic ADAMs, namely ADAM 10 and 17. ADAM 10 cleaves pro-TNF-α, notch and type-IV collagen, while ADAM 17 activates TNF-α, transforming growth factor-α and Notch. Both of them are important sheddases that anchored onto plasma membrane surface to activate signalling messengers and released their substrates from the trapping surface. However, the substrates that can be processed by the spermatogenic ADAMs are still not clear. It is also known that not every member of ADAM and ADAMTS owns protease activities. Bioinformatics and sequence analysis suggest that these family of genes share extensive homology with MMPs, in which MMPs are already well-established for their roles in matrix remodelling. The use of labelled zymogram analysis may help to understand the nature of substances that are substrates of these potential proteases.

~ Another question to understand the role of ADAMs and ADAMTSs to male fertility is that the cellular counterpart of the adhesive domains in these molecules is not known. The only well-studied case is the involvement of ADAMs 1 to 3 in fertilization. The ADAM1a/ADAM2 heterodimer together with ADAM3 are directly involves in the sperm penetration and zona pellucida binding through their ability of adhesive domains to bind with integrins from female reproductive tract and oozyte. Another proven candidate of ADAM members that can interact with integrin subunit is ADAM15, which have been shown to involve in interactions with matrix $\alpha 9\beta 1$ integrin in many cases like cancer and cartilage remodelling. Even though the composition of testis-specific ectoplasmic specialization is largely unknown at the molecular level, $\beta 1$ integrin is though to be one of the main components within these junctions[125]. ADAMs are supposed to act as a platform for shedding of biological active factors to support cell adhesion, while integrins are the most promising candidates[126]. Thus ADAMs may be involved in the site-directed matrix remodelling or membrane fusion, where the disintegrin or EGF-like domain binds to specific locations first; upon cleavage of pro-domain, metalloprotease domain is activated for other purposes of the cellular machineries.

Localization of these genes are only predicted from micro-array data obtained by physical isolation of cells, extensive investigation at protein levels, for example immunohistochemistry and/or *in situ* hybridization are required to verify their presence at cellular level. Localization of these ADAMs in the testis can give us a picture on how neonatal hypothyroidism affects the assembly of testicular junctions and spermatogenesis. As shown previously, overall expression of genes may not be affected, but the trafficking and distribution of gene expression may be influenced. Just like the junction proteins like Cx43 or occludin, ADAMs are cell surface

molecules that only be functional when they are anchored onto the plasma membrane. It is known that some ADAMs are only found in meiotic spermatocytes, while the others are post-meiotic spermatid specific. Co-localization together with junctional complexes, for example tight junction or ectoplasmic specialization in specific, may give us some clues in proving ADAM's ability in matrix or junction remodelling in the testis. ADAMTSs and many MMPs are soluble and secretory enzymes that may not have a specific localization. Thus knowing their cellular locations, especially on the spermatogenic members, may help to characterize and understand their involvements in spermatogenesis. Knowing more on relation of these molecules to the expression of Sertoli cell, Leydig cells and germ cells specific regulatory genes are probably helpful for us to know how neonatal hypothyroidism induced testicular hypertrophy in adult rats.

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